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VIRAL MEDIATED GENE TRANSFER IN A LARGE ANIMAL HUMAN GLIOMA MODEL

BY

SIVASUPIRAMANIAM SRIHARAN

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE**

IN

EXPERIMENTAL SURGERY

DEPARTMENT OF SURGERY

EDMONTON, ALBERTA
FALL 1994

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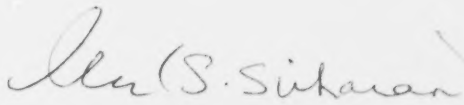

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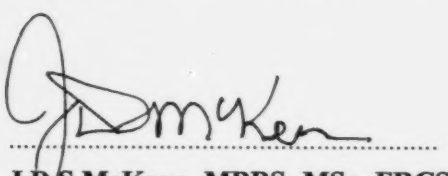
The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **VIRAL MEDIATED GENE TRANSFER IN A LARGE ANIMAL HUMAN GLIOMA MODEL** submitted by **SIVASUPIRAMANIAM SRIHARAN** in partial fulfillment for the degree of **MASTER OF SCIENCE** in **EXPERIMENTAL SURGERY.**

K.C.Petruk, M.D., FRCS(C), PhD., supervisor



J.D.S.McKean, MBBS, MSc, FRCS(C), DABNS

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ABSTRACT

Adenoviral mediated gene transfer into human gliomas was assessed both *in vitro* and *in vivo*. A genetically engineered adenovirus(serotype 5 virus) carrying a reporter gene, the E.coli lacZ gene, was used as the viral vector. The D-54 MG cell line as well as 6 other cell lines derived from our own clinical patients were used for the *in vitro* studies. The cell lines were grown in culture to confluence and subsequently incubated with 1000 plaque forming units(pfu's) per cell of the experimental adenovirus for 24 hours. They were then treated with X-gal solution, which acts as a substrate for the gene product β–galactosidase. The sections were then assessed under the high power of a light microscope for the percentage of blue, transduced cells.

The *in vivo* studies were carried out on a large animal human glioma model which had been previously developed in this laboratory. Cats, chronically immunesuppressed with Cyclosporin A, were implanted intracerebrally with D-54 MG cells. Once a tumor was seen on MRI imaging studies, the tumor was innoculated with the experimental adenovirus. The animals were euthanized after 48 hours and the brain fixed in vivo. Cryostat sections were incubated with the X-gal substrate and subsequently assessed for the percentage of transduced tumor cells.

90-100 % transduction of all 7 glioblastoma cell lines was seen *in vitro*. 70-100% transduction was seen, extending for a distance of 500-600μM circumferentially around the site of injection. 90-100% transduction was seen in areas of tumor growing along leptomeningeal surfaces. This transduction rate was maintained over a larger surface area. Negligible areas of normal brain were transduced. There was no clinical evidence of neurotoxicity secondary to the viral injection.

Thus, the adenovirus is an efficient agent for the transfer of genes into human gliomas. Biodistribution, however, remains a short-coming of the current vector and/or the current mode of viral delivery.

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Chapter 1. Introduction

The Biology and Contemporary Treatment of Malignant Gliomas

Brain cancer remains a debilitating and rapidly fatal disease. Both the incidence and mortality of this disease have been increasing in most industrial countries, mostly in the elderly, since the mid 60's(1). One fourth of all childhood cancer deaths are due to brain cancer. The incidence of gliomas varies worldwide from 1 to 15 per 100,000(2-4) , the variability due in part to the availability of technology. The median age adjusted annual incidence of gliomas varies between 2.5 and 5.5 cases per 100,000 population / year (5-10). Gliomas constitute 40 to 60% of all intracranial tumors. More than 90% of primary malignant CNS tumors are malignant gliomas(11).

The relative frequency of histologic types of brain tumors in children differs markedly from that in adults; the location of tumors also varies. Tumors in the first year of life are usually supratentorial, but between 2 and 12, 85% of the tumors are infratentorial(12). Supratentorial tumors increase in frequency during the teenage years and predominate in adults. In most cases a bimodal age distribution in incidence occurs with an early peak at 0 - 5 years and a later peak at 60 - 65 years. The timing of the peak incidence in old age varies and has been shifting from age 45 - 65 to 65 - 75 in recent years(13-15). Modan et al(16) proposed several explanations for these increases.

(1) Increased exposure to a newly emerging carcinogen or decreased exposure to a protective factor.

(2) Delayed reaction to an increased carcinogenic exposure early in life with an extremely prolonged latency period.

(3) Changes in disease classification.

(4) Improved diagnosis with the advent of CT and MRI scans.

(5) Higher probability for brain cancer to be listed as the underlying rather than the contributory cause of death.

(6) Changes in health care delivery to the elderly. Modan et al showed that diagnostic improvements were the most likely explanation for increased rates in the elderly.

The male: female ratio in recently reported series has been 1.7 : 1. These sex differences may be due to differences in access to health care or differential exposure,

susceptibility or resistance to neuro-oncogenic agents(17). Variation in the sex ratio by histology type suggests differential effects on the hosts susceptibility to some carcinogenic agents.

Lower rates of brain cancer occur in Asia and Africa than in Europe and the USA. In the USA, rates are usually increased for Whites than for Blacks, Asians or Hispanics(11).

Etiology

Numerous environmental/lifestyle factors have been considered in the etiology of brain cancer. Certain chemical carcinogens and ionizing radiation have been shown convincingly to play a role in the etiology of brain tumors. For many other factors the evidence is weak, inconclusive, or clearly negative.

Radiation

Ron and Modan(18) showed that Israelis treated for ring worm(tinea capitis) as children were four times likely than controls to develop malignancies (10/10,842 vs 4/16,242). A Swedish study showed an increased incidence of brain tumors close to sites of radiation for skin haemangiomas(19). Another series of patients with glioblastoma showed a high prevalence of previous therapeutic radiation(20). Epidemiological studies have shown increased rates of second primary tumors in survivors of childhood brain cancer exposed to radiation for treatment of their first malignancy(21-24).

Chemical Agents

Certain chemicals can induce brain tumors, at least in experimental models(25). Gliomas have been induced by polycyclic aromatic hydrocarbons and by IV administration of nitroso compounds. Others include hydrazo, azo and azoxy compounds, acrylonitrile, vinyl chloride and ethylene oxide. Workers exposed to polyvinyl chloride(PVC), formaldehyde, pesticides and petroleum and petro-chemical products have all been shown to have an increased risk of brain tumors(26-30).

Hereditary Influences

Various hereditary syndromes are associated with an increased incidence of brain tumors, especially neurofibromatosis type I and nevoid basal cell cancer syndrome(7, 31-36).This is detailed in the table below and is discussed in more detail in the section 'Genetic Alterations in Brain Tumors'.

Hereditary Syndromes Associated with Brain Tumors

Hereditary condition	Type of CNS Tumor	Chromosome Involved
Tuberous sclerosis	Ependymoma; astrocytic glioma; ganglioneuroma; glioblastoma	9q32-34 or 11q
Von Recklinghausen neurofibromatosis (NF 1)	Glioma (optic nerve)	17q12-17q22
Bilateral acoustic neurofibromatosis (NF 2)	Schwannoma; meningioma	22q
Nevoid basal cell carcinoma syndrome	Medulloblastoma	1q22 9q31
Turcot syndrome Gardner's syndrome Familial polyposis	Diverse histologic types of brain tumor including glioblastoma and medulloblastoma	5q
Sturge-Weber syndrome	Choroid plexus	Not known
Von Hippel-Lindau disease	Hemangioblastoma	3p13-14 3p25-3p26
Li-Fraumeni syndrome	Glioma; medulloblastoma	17p(p53)

Histology

There are various grading systems for astrocytomas and to a lesser extent other gliomas. Kernohan's Gd I - IV correspond to the WHO classification Gd II - IV. A more recent system termed the St Anne-Mayo system(or Damas-Duport)(37) simply records the presence of nuclear atypia, mitoses, endothelial proliferation and necrosis, without weighing the significance of particular combinations.

There is a spectrum from well-differentiated through anaplastic astrocytoma to glioblastoma. Features suggesting anaplastic transformation include increased cellularity,

nuclear atypia, cellular pleomorphism, mitotic activity, endothelial or vascular proliferation and necrosis.

Tumors are graded as follows:

Gd1 - none of these features.

2 - one

3 - two

4 - three or four criteria.

The system is easily applied, reproducible and shows close correlation with patient survival. In general Gd 2 tumors show nuclear atypia, Gd 3 lesions show addition of mitoses and Gd 4 tumors further show endothelial proliferation and or necrosis.

A revised WHO classification, however, is the most accepted system at present. Gliomas are classified as follows based on the presence of various combinations of the previously mentioned features such as nuclear atypia, presence of mitotic figures, endothelial proliferation, etc.

Grade I	Low grade (former grades I and II)
Grade II	Anaplastic astrocytoma
Grade III	Glioblastoma multiforme

Cell Cycle Markers

The incorporation of tumor kinetics in the routine assessment of histopathological grade is proving increasingly useful in evaluating the proliferative potential of central nervous system tumors.

Cell cycle markers such as the silver nucleolar organizer region or AgNOR technique, immunohistochemical methods such as Ki-67 and proliferating cell nuclear antigen(PCNA) and *in vitro* and *in vivo* uptake of bromodeoxyuridine (BrdU) help predict the biological aggressiveness of tumors(38-41).There are various ways of evaluating cell kinetics. The mitotic index is an approximately accurate and restrictive method. The labeling index after [^{3}H] thymidine correlates with prognosis.

Administration of BrdU, revealed by monoclonal antibody, to either patients or small fragments of tumor in culture may be more precise as it labels cells in S-phase. A more recent version can be applied in vitro to biopsy samples from patients using a monoclonal antibody assay(42, 43)

Reaction of the monoclonal antibody Ki-67 with a nuclear antigen expressed by cells in cycle gives an indication of mitotic rate but it has been shown that not all dividing cells express this antigen.

The neoplastic transformation of glial cells to astrocytomas

The cell of origin of astrocytomas is still disputed. There is controversy as to whether neurons and glial cells are produced by the same germinal cells, by different, but histologically indistinguishable cells or from the same cells consecutively(44, 45).

In the early stages of development, the neural tube is composed of asynchronously proliferating germinal cells. A few days later the cells enter the post-mitotic phase and, as young neurons migrate from the germinal to the marginal zone, which eventually becomes the anlage of the pallium. Glial cells migrate later retaining their proliferative capacity while doing so. The subventricular and intermediate zones will become the white matter and contain mitotic glial cells. Neurons cease to proliferate on migration. The point of divergence of glial and neuronal cell lines is still disputed. His(46) postulated two cell lines in the germinal layer - the spongioblast and germinal cells. Schaper(47) postulated a single, mitotically active population producing "indifferent" cells which migrate into the mantle layer and give rise to both neurons and glia. Recent evidence however suggests that neurons and neuroglial precursors must co-exist in the initial stages of development. Glial cells however are not produced until the production of neurons ceases. This temporal dissociation is borne out by experiment carcinogenesis.

The histogenetic relationship between astrocytes and oligodendrocytes is still uncertain and whether the two types arise simultaneously or consecutively and whether from the same or different precursors(48, 49).

Gliogenesis in the adult animal is not fully clear. Do new glial cells originate from stem cells or is DNA synthesized in terminally differentiated cells ? The subependymal plate is the main site of glial cell production. However, stem cells are also found in the adult myelination glia, outer granular layer of the cerebellar cortex, fascia dentata, and molecular layer. One third of the subependymal plate cells are in cycle compared with 1% of glial cells. Thus late fetal neuroepithelial cells and the above 5 sites are putative neoplastic transformation sites. Neoplastic transformation is also a multi step process with shifting of cells between strikes with the result that the tumor may arise elsewhere than the site of first strike, for example in the cerebral cortex.

There is a window of neoplastic vulnerability during which cells are susceptible to transformation. The width of this window depends on, among other things, the existence of a reservoir of stem cells, the ability of differentiated cells to re-enter the cycle, the number of duplicating cells at risk, the length of the period in which the cell population is in the cycle and it's differentiation status.

The progression from benign to more malignant forms is an important concept. An astrocytoma grows slowly, is composed of isomorphic cells and has poor angiogenesis. At a certain point in its biological course, however, it may change its rate of growth and become malignant. Cells pass from the non-proliferating to the proliferating pool increasing the growth fraction , leading to anaplasia. This is seen morphologically as an increase in cell density and mitosis, the appearance of circumscribed necroses and angiogenesis. Anaplasia can be regarded as either dedifferentiation, i.e., loss of morphological characteristics typical of a certain degree of differentiation, with regression to those of a more primitive stage or, as a failure of differentiation whereby tumor cells do not reach morphological maturity.

Recent thinking however considers anaplasia as an expression of the heterogeneity of the tumor cell population. The expression of GFAP, a characteristic marker of astrocyte differentiation, is found in glioma cells with gliofibrillogenetic capacity and the number of positive cells is inversely related to the degree of anaplasia. The growth fraction increases as cells are recruited from the non-proliferating to the proliferating pool. Clonogenic populations will develop and be selected out by competition. Thus tumor progression is a function of both genetic instability and environmental selection.

The Cell Cycle

Tumor development is to a large extent determined by the activation of oncogenes and/or loss of tumor supressor genes. In order to fully appreciate this, the control of the cell cycle will first be described.

The cell cycle is divided into four phases G1, S, G2 and M as shown in the diagram below. Those factors within the circle are essential for cycle progression; without these functions the cycle fails to progress and a mutation is lethal.

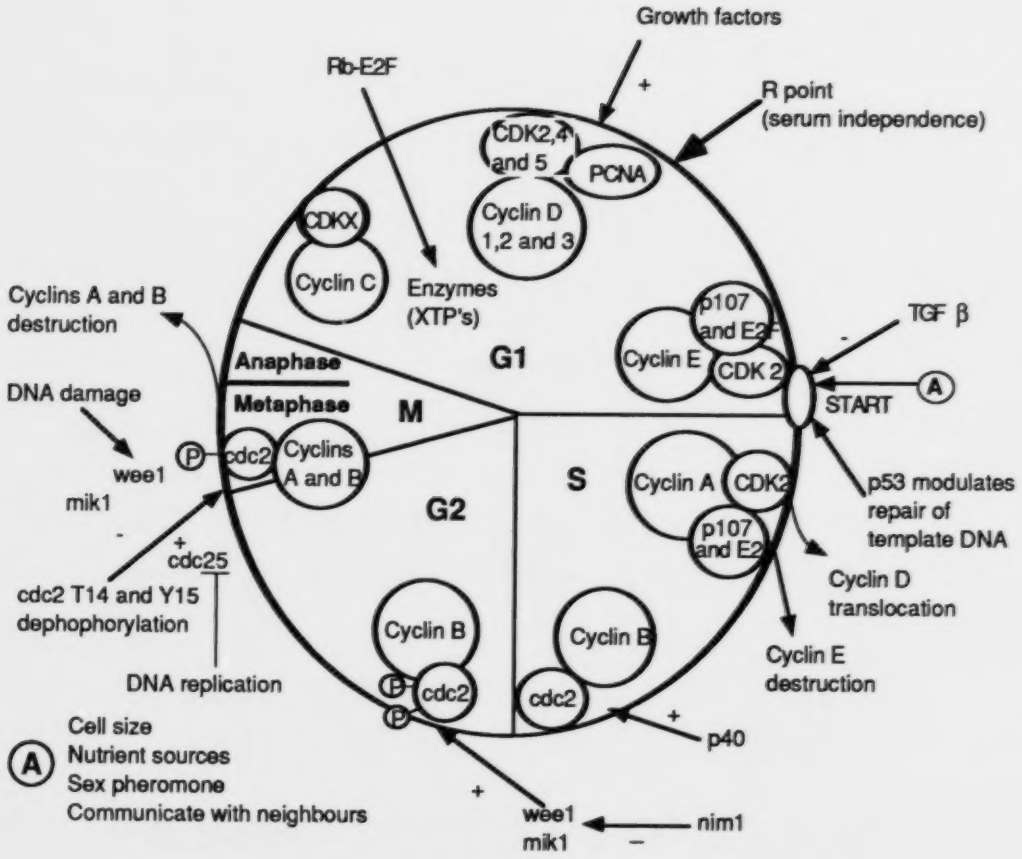
The check points on the outside of the circle are non-essential but help regulate the cell cycle. They negatively regulate the cycle, are not lethal to a cell if absent but lead instead to an inefficient cell cycle resulting in genomic instability, mutation and neoplastic transformation of the cell. Check points utilize signal transduction pathways to regulate the essential functions in the cell cycle. Some check points are tumor suppressor genes and some factors in the signal transduction pathway are proto-oncogenes.

Obligatory functions include:

G1- synthesis or activation of enzymes in late G1 required for deoxyribonucleotide triphosphate precursors for DNA synthesis in S-phase.

S- synthesis of DNA polymerases, ligases, primases, helicases, single strand DNA binding proteins , histone synthesis and DNA packaging required in S-phase to progress through cell cycle.

G2- chromosome condensation; spindle attachment and segregation.



Check point controls monitor various things and modify the cell cycle accordingly. They monitor nutrient levels; if found inadequate the cell cycle is blocked in

G-1. Another G-1 check point measures if the DNA template is normal or damaged; if this is found the cycle is shut down in late G-1 till DNA repair occurs.

One of the most critical check point in G-1 is called *start* in the yeast cell cycle and the *restriction point* in mammalian cells. Beyond this point cells will progress through the cycle even in the presence of inadequate nutrition, etc.

Platelet derived growth factor (PDGF) and epidermal growth factor(EGF) promote cycling; removal prior to reaching the restriction point stops the cycle. Once removed after passing this point, there is no effect on progression through the cycle.

The S-phase check point is RCC 1 in humans. It monitors the DNA to ensure the S-phase is complete before the cells enter G-2 and M-phase. RCC 1 prevents M-phase condensation factor production until all the DNA is replicated in S-phase. RAD 9 (6 genes) check point in G-2 prevents cells from entering mitosis with damaged chromosomes. It also helps coordinate events in S-phase with mitosis.

Each of these check points ensures that a cell with damaged DNA does not propagate. Aberrant cell cycles result in chromosomal translocations, amplifications and aneuploidy.

Cell cycle aberrations may also result from abnormal levels of expressions of certain essential functional genes e.g.. histones synthesis. These and the MIF 2 gene in yeast are essential genes (lethal if deleted) which act as structural elements in essential cell cycle events (not as negative down-regulators of downstream events), but the result, disruption of the efficiency of cell cycle processes, is the same as for check point functions. The abnormal expression levels of critical genes can lead to cell cycle aberration. One such critical gene is the *myc* oncogene which is commonly expressed in abnormally high levels in tumor cells (due either to chromosomal translocation , promoter activation by integration of a viral LTR sequence or via gene amplification).

Activated oncogenes can override check point controls. Check points produce their effects in part via proteins called cyclins which activate catalytic subunits of protein kinases(p34 cdc kinases). The cdc 2 protein is a catalytic subunit of a protein kinase that phosphorylates serine residues in a serine-proline amino acid sequence.

Cyclin proteins (cyc B) form a tight protein complex with p34 cdc 2 kinase and when active send a strong signal to enter M-phase and trigger mitosis. Cyclin levels and activity are regulated by environmental stimuli via a number of gene functions.

1) a receptor

2) a G protein transducing signal

3) a series of protein kinases

4) several transcription factors that regulate cyclin synthesis

5) some factors that regulate cyclins in a post translational mode

Several cyclins have now been identified (cyclins A, B1, B2, C1, D and E) and function in G1 (cyclin C at the restriction point) and G1/ S (cyclin E at the G1/S border). There is also a rapidly growing family of p34 cdc 2 like kinases. This results in a large number of combinations, i.e., number of enzyme activities.

Growth hormones, growth inhibitory signals, G-proteins, protein kinases, phosphatases, proteases and transcription factors all play a role in the signal transduction pathways between check point pathways and essential events in the cell cycle. The cyclin- cdc 2 complex is further regulated by phosphorylation of the cdc protein. Several kinases (mcc 1, mik 1) and phosphatases (cdc 25, stf 1) regulate the activity of the cyclin- cdc 2 kinase in this post-translational manner.

Oncogenes

Many of the linkage functions in the signal transduction pathway are mediated by oncogenes. The retinoblastoma susceptibility gene (RB1) and p53 are both tumor suppressor genes. These genes are not essential for the life of the cell and normal cell division can proceed in the absence of RB1 and p53. Loss of the Rb gene in humans gives rise to retinoblastomas and predisposes to osteosarcoma.

Loss of the p53 gene predisposes to a variety of cancers. Mutations in the p53 gene contribute to the development of up to 50% of human cancers. p53 has a similar function to the RAD 9 gene function. Gamma radiation of cells with normal wild type p53 protein results in a pause or block in G1 prior to entry to the S-phase. Cells with no p53 protein fail to pause in G1- phase, enter S-phase and die at much higher rates than cells with normal p53 protein. DNA damage by UV or gamma radiation induces high levels of p53 by some post-translational mechanism that appears to stabilize an otherwise unstable protein. p53 acts as a late G1 check point making sure that the DNA template is intact and ready for normal DNA replication in S-phase.

The normal p53 gene protein assembles into homotetramers and higher order homo-oligomeric complexes. Mutant forms may form a multi- subunit which cannot function.

Viral oncoproteins such as the SV40 large T-antigen and E1B sequester p53 in an inactive complex thus preventing its normal action in cell cycle regulation. The HPV E6-oncoprotein by associating with the p53 molecules tags them for rapid destruction unlike the Rb protein.

The p53 protein does not appear to undergo massive, cyclin-driven phosphorylation like pRB. Two signals have been implicated in modulating p53 function.

1) A generational clock which uses p53 to shut down proliferation when a cell lineage has exhausted its allotted number of division cycles.

2) p53 protein is involved in regulating DNA repair following damage.

The p53 does not actively survey genomic integrity; instead p53 responds to signals sent out by other genomic monitors.

More recently it has been shown by three different groups(50-52) that the p53 gene product stimulates the production of another protein, the p21 protein(21kD) which is coded for by the Cip 1/WAF 1 gene. The p21 protein blocks the action of the Cdk/cyclin complexes, especially Cdk 2. The cyclin activated Cdk 2 normally prepares cells for cell division by pushing them out of the first growth phase of the cell cycle into the DNA-synthesizing phase. Thus a p53 mutation actually causes a decrease in the production of the p21 protein resulting in uncontrolled and especially unmonitored cell division.

The Rb protein negatively regulates a transcription factor E2F that in turn plays a role in regulating enzyme activities that contribute to nucleoside triphosphate and DNA synthesis.

Thus it can be seen that alterations or perturbations of the cell cycle can result in uncontrolled cell proliferation , leading eventually to the development of neoplasia.

Genetic alterations in human gliomas

Malignant progression of astrocytic tumors has been correlated with a sequential accumulation of genetic alterations. These include both activation of oncogenes which are activated or aberrant forms of normal cellular genes(proto-oncogenes), the inhibition of various tumor suppressor genes as well as the stimulation of various growth factors.

Tumor suppressor genes

The p53 gene

The loss of alleles on the distal, short arm of chromosome 17 including the p53 tumor suppressor gene may be important in tumor initiation and/or progression as it is seen in both high and low grade tumors.

40% of low and intermediate grade tumors and 30% of high grade tumors show these losses(53-57).Loss of genetic information from the short arm of chromosome 17 is found in 5 of 19 low grade gliomas, 7 of 24 anaplastic astrocytomas and 19 of 49 glioblastomas(53, 55, 57). There may be another as yet unidentified tumor suppressor gene residing on chromosome 17 besides p53(58-60). Several groups have shown that loss of one p53 allele is usually associated with a point mutation of the remaining p53 allele(58-60). Germline p53 mutations predispose to the development of glial tumors. This is seen in the Li-Fraumeni syndrome and in many patients suffering from multi-focal gliomas or with a glioma and a second primary malignancy.

There is a suggestion that clinically, glioblastomas containing p53 mutations may represent a subgroup of tumors with an earlier age of onset and better survival(61).

With reference to p53 there may be two or more distinct genetic pathways in the development of malignant gliomas(62).

(i) De-differentiation of a low grade glioma to a high grade one in a subgroup of younger patients with p53 mutations.

(ii) A de novo pathway results in the direct development of high grade tumors in older patients without p53 mutations.

The Retinoblastoma Susceptibility Gene

The RB1 gene may also be altered in gliomas. Loss of markers on chromosome 13 within the RB1 locus is seen in 13-25% of astrocytomas, mainly glioblastomas. There is a lack of RB1 mRNA in several glioma cell lines suggesting a suppression of RB1 transcription in tumorigenesis(63).

Other putative suppressor genes

25% of high-grade astrocytomas show loss of the distal portion of chromosome 11p, i.e. distal to the Wilm's Tumor susceptibility gene(64).

The NF1 gene is a tumor supressor gene which codes for neurofibromin. Loss of this gene results in numerous tumors including anaplastic astrocytomas(7, 32, 65, 66).A 360 amino acid domain of neurofibromin shows sequence homology to the catalytic domain of the mammalian p120 GAP(p21ras - GTPase - activating protein) and the yeast IRA1 and IRA2(inhibitors of the ras - cyclic adenosine monophophate[AMP]-activating pathway 1 and 2)protein. The membrane associated p21ras protein binds GTP in the active state. GAP and related proteins accelerate the hydrolysis of GTP to GDP thus inactivating p21. Altered splicing results in variant GAP-related NF1 domains, Type I and II NF1-GRD(67, 68).Nearly all glioblastomas express more type I transcript than

type II. This ratio is reversed in normal brain. The significance of this differential expression is as yet undetermined.

Turcot syndrome is characterized by multiple intestinal polyposis and neuro-epithelial tumors. Therefore one or more tumor suppressor genes associated with intestinal polyposis such as APC, MCC and DCC may be involved in gliomagenesis(33).

Chromosome 10

80% of glioblastoma multiformes show loss of alleles on chromosome 10(69).There is a highly specific association of loss of genetic information from chromosome 10 and glioblastomas, being present in 59 of 84 glioblastomas, 5 of 33 anaplastic astrocytomas, and 0 of 33 low grade gliomas(55, 70-72). Most glioblastomas lose an entire copy of chromosome 10, while a smaller number show partial loss and a few have a small regional loss. A putative tumor suppressor gene may exist on the distal long arm of chromosome 10(also implicated in prostate cancer and melanomas) and a second one on 10p(73, 74).

Chromosome 9

Loss of alleles on 9p occurs in 30-40% of glial tumors (75).The interferon α and β gene loci are found in the deleted portions(76).It may also contain a tumor suppressor gene which may be involved in tumor progression as all the tumors showing loss of the interferon genes were either anaplastic astrocytomas or glioblastomas.

Loss of one allele of the Type I interferon(IFN-β and/or IFN-α)gene is mostly seen in Grade III tumors whereas loss of both alleles occurs only in glioblastomas(76). It would therefore seem that tumor cells try to eliminate those genes whose products inhibit their growth. This has been partly borne out by the fact that transfer into and expression of an IFN gene into cells of cultured gliomas results in marked growth inhibition(77).

In melanomas , however, a gene in close proximity to the Type I IFN loci has been implicated in tumorigenesis; the same may be true in a high percentage(88%) of gliomas(78).

Other changes

A small (10%) subgroup of high grade tumors show loss of alleles on the distal arm of chromosome 22q. Genetic loss was determined in 2 of 12 low grade gliomas, 2 of 10 anaplastic astrocytoma and 5 of 15 glioblastomas.

Loss of genetic information from chromosome 13 was seen in two of 13 low grade gliomas 2 of 9 anaplastic astrocytomas and 4 of 14 glioblastomas. Loss of the sex

chromosomes has also been noted in both high grade and low grade tumors. However, this is also seen in non-neoplastic brain tissue in short term culture(69, 79).

One study(80)has shown that cytogenetic analysis provides independent prognostic information in patients with cerebral astrocytomas. Patients with tumors showing normal or nonclonal karyotypes faring better than those with clonal abnormalities.

Growth Factors

Numerous growth factors are found to be over-expressed in malignant gliomas. They help provide various factors and alter the surrounding milieu such as to promote glioma growth. Growth factors stimulate tumor growth and/or angiogenesis by forming autocrine and/or paracrine stimulatory loops with appropriate receptor bearing cells.

Epidermal growth factor receptor

Amplification of the EGFR gene(EGFR/c-erbB1) occurs in 40-50% of glioblastomas and correlates with the over-expression and with the presence of *double minutes.* However gains of chromosome 7 do not seem to correlate with EGFR amplification and other tumor related genes on chromosome 7 are not co-amplified with EGFR.

The EGFR gene product is a tyrosine kinase with an extra-cellular ligand binding domain which normally binds EGF and TGFα.The genes for EGF and/or TGFα are expressed along with normal or amplified and rearranged EGFR's in high-grade and low-grade glial tumors. Thus EGF and TGF-α acting through EGFR may form autocrine and/or paracrine growth stimulatory loops(81, 82).

3 types of EGF-R are produced by gene rearrangements and deletions in the extracellular domain. The hybrid transcripts all retain the native reading frame of the receptor suggesting that preservation of some of the functions of the EGF receptor is essential for tumorigenesis(83-85).

The Type I receptor has a low level of kinase activity in the absence of growth factor(86).

The Type II receptor has kinetics similar to the normal receptor(87).

The Type III receptor, which is the commonest result of EGFR gene amplification carries a deletion in the extra-cellular ligand binding domain. These mutant receptors fail to bind EGFR and instead demonstrate constitutive tyrosine kinase activity. The Type III receptor cDNA when cloned into NIH 3T3 fibroblasts causes morphologic

transformation , enhanced cell growth and high cloning efficiency in soft agar(84). This occurs in the absence of EGF suggesting constitutive tyrosine kinase activity.

A less common rearrangement at the 3' end of the EGFR gene may result in faulty internalization and/or degradation of EGF-R.

EGFR amplification occurs in a variety of tumors and is associated with a poorer prognosis in other solid tumors(88).The correlation in gliomas has been variable(69, 89, 90). EGFR amplification occurs exclusively with loss of chromosome 10 suggesting an invariable association between the two genetic events.

Other tyrosine kinases

The HER-2/neu(c-erb B2 or $p185^{erb\ B2}$) proto-oncogene is a receptor tyrosine kinase with homology to EGFR. A point mutation in its trans-membrane domain results in transforming activity. Ligands for this erb B2 receptor include the ***heregulins*** as well as glial growth factors I - III. The gene for the latter also codes for acetylcholine receptor - inducing activity and for the heregulins as a result of alternate splicing.

The glial growth factors I - III are trophic for Schwann cells produced by glial tumors and may form growth stimulatory autocrine and/or paracrine loops with the erb B2 receptor thus helping in oncogenesis.

Platelet derived growth factors

Platelet derived growth factors may play a role in glioma genesis because:

(i) Brain tumors may be induced in marmoset monkeys by intracerebral injection of simian sarcoma virus. The v-sis oncogene of this virus is the retroviral homologue of the PDGF-B chain(91).

There are two separate genes a and b which code for A and B chains. Homo or heterodimerisation of these chains via disulfide bonds results in 3 isoforms, PDGF-AA, PDGF-AB and PDGF-BB(92).The PDGF-α receptor binds all 3 isoforms with equal affinity. The PDGF-β receptor binds PDGF-BB with high affinity and the other 2 with lesser affinities.

Gene amplification with over-expression of the PDGF-α receptor occurs in glial tumor cells(4 of 50 glioblastomas) while the PDGF-β receptor is found in the hyperplastic endothelial cells within tumors(91) but no over-expression or gene amplification of the b chain has been seen(93). Binding of PDGF to its receptor activates tyrosine kinase activity resulting in a cascade of intra-cellular events.

Other growth factors

As tumors grow they require angiogenesis to provide adequate nutrients(94). Several endothelial factors show angiogenic activity including fibroblast growth factors (FGF's), PDGF and vascular endothelial growth factor(VEGF/vascular permeability factor).

Basic FGF is over-expressed in high-grade gliomas compared to low grade ones whereas acidic FGF is more frequently expressed in astrocytic tumors than in meningiomas(95).

VEGF is a potent angiogenic molecule and vascular permeability factor. VEGF mRNA is over-expressed by 50-fold in glioblastomas compared to astrocytomas. Induction of *flt* ,the VEGF receptor, occurs in the tumor vascular endothelial cells(96). VEGF mRNA is localized to tumor cells whereas the VEGF protein is found exclusively in the adjacent endothelial cells. This suggests that VEGF may mediate angiogenesis *in vivo* through paracrine stimulatory loops involving the secretion of VEGF by the tumor cells and the stimulation of endothelial cells bearing VEGF receptors(96, 97). VEGF seems to be highly induced in glial cells immediately adjacent to necrotic foci thus implicating hypoxia as a stimulus for its secretion(97).

Oncogenes

N-myc and *gli* oncogenes have also been found to be amplified in 5% of glial tumors(98).

The *gli* gene on chromosome 12 is a member of the zinc-finger binding protein family of DNA binding proteins. It was found to be highly amplified in one malignant glioma(99).No further mention of this gene being amplified in gliomas can be found in the literature. Its significance therefore remains uncertain.

The *ros 1* gene shows high levels of expression in several glioblastoma cell lines and gene re-arrangement in one cell line(100). It encodes for a protein of unknown function but with homology to transmembrane receptor kinases such as EGFR. In vivo studies looking for *ros 1* gene amplification have given mixed results (101).

The MDM 2 gene(murine double minute 2) codes for a cellular protein which can complex with the p53 gene product to inhibit its function(102). 8-10% of anaplastic astrocytomas and glioblastomas show amplification and over-expression of the MDM 2 gene. Therefore even tumors not showing obvious p53 abnormalities could still have defective p53 function as a result of this interaction(103).

Occasional amplification and/or gene rearrangement of *c-myc, e-myc, c-abl and k-ras* have been reported in cell lines or xenografts of glioblastomas but too inconsistently to be of importance in the pathogenesis of malignant gliomas.(98, 104)

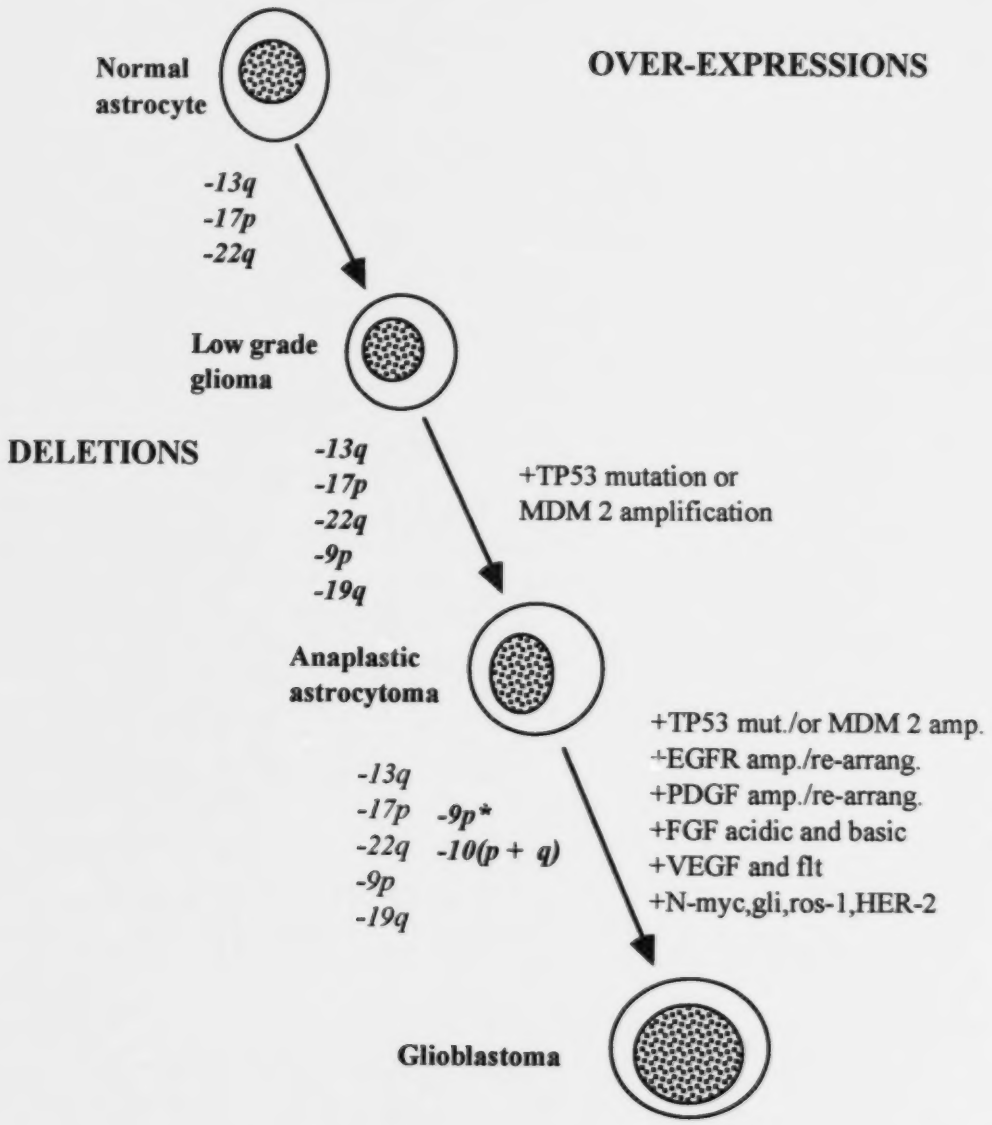
Summary of genetic changes in glioma induction and transformation

The earliest event in glioma progression includes loss of genetic information from the long arms of chromosomes 13 or 22 or the short arm of chromosome 17.

Loss of a single complement of the Type I interferon(IFN) gene from 9p and loss of genetic information from 19q characterize tumors of intermediate malignancy.

The most malignant glial tumors show loss of the second Type I IFN gene complement, loss of genetic information from chromosome 10 and gene amplification (in 40% of cases this was EGFR).

The flow diagram on the next page summarizes all the known changes that occur in the development of a glioblastoma. Multiple gene alterations take place during this process . Targeting of single genes for modification could therefore not hope to effect a cure.



The schema shown above details the known genetic changes that occur during the transformation of a precursor cell into a glioblastoma multiforme. The changes depicted to the left of the flow diagram constitute the many gene/chromosomal deletions that occur during this process while those detailed on the right side represent amplification and/or over-expression of various genes.

Immunobiology of brain tumors

The CNS has been considered an immunologically privileged site with limited immunoreactivity. This view has been increasingly challenged over recent years. Brain tumors subvert the host immune system in order to escape detection and attack. Normalization or augmentation of the host immune responses may therefore result in rejection of the tumor.

Endothelial cells express cell surface molecules and produce various cytokines that help signal the immune system resulting in the movement of cells across the blood-brain barrier.

The cervical lymphatics and dural sinuses collect both large and small molecules respectively that leave the CNS via the interstitial fluid and CSF.

This immunoreactivity, however, is countered by brain cells resulting in dampening of both Ag(antigen)-specific cell mediated immunity and Ab(antibody) responses. There is also limited expression of Class 1 MHC molecules on brain cells.

T-cell function

T-cell function is suppressed in patients with glioblastoma multiforme (GBM)(105). This includes:

(1) a decrease in the number of peripheral blood T-lymphocytes

(2) impairment of mitogen and Ag-mediated T-cell reactivity

(3) decreased response of T-cells to phytohemataggluti nin

(4) decreased production of IL-2

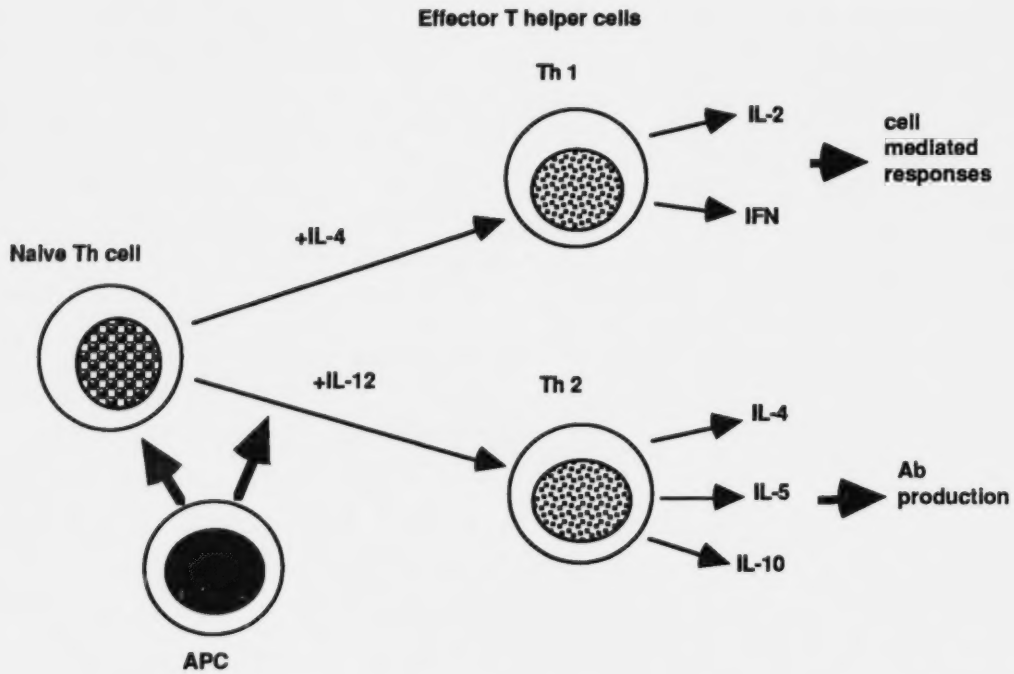
(5) decreased expression of the IL-2 receptor p55 chain(Tac.α-chain)

The reduced numbers of circulating T- cells is due to a selective depletion of Th (T-helper) cells(106). Th cells are important for both cell mediated and antibody mediated immune responses as shown in the figure below. Thus a depletion or functional impairment of Th cells would result in a greatly dampened immune response.

Patients with GBM have normal B-cells and serum immunoglobulins(Ig's). However, they have a decreased Ab response to tetanus, influenza and other antigens. This may be secondary to defective Th cell function.

Very few Th and B-cells are found in the population of tumor infiltrating lymphocytes(TIL's). Thus the production of anti-glioma antigens is limited.

GBM's produce IL-6 which is a potent B-cell stimulator; they also produce TGF β-2 which is a potent inhibitor of IgG and IgM secretion by B-cells(107). Ab-mediated inflammatory reactions are also hampered by low concentrations of complement components in brain CSF.



Tumor Associated Antigens

Tumor associated antigens(TAA's) are antigens expressed on tumor cells which have the potential ability to evoke rejection of a tumor in immunized hosts. There has only been one successful trial of active immunization with human glioma cells(108).This was tested in two rat models of RT2 and C6 gliomas(109, 110).The TAA provoking this response has yet to be identified.

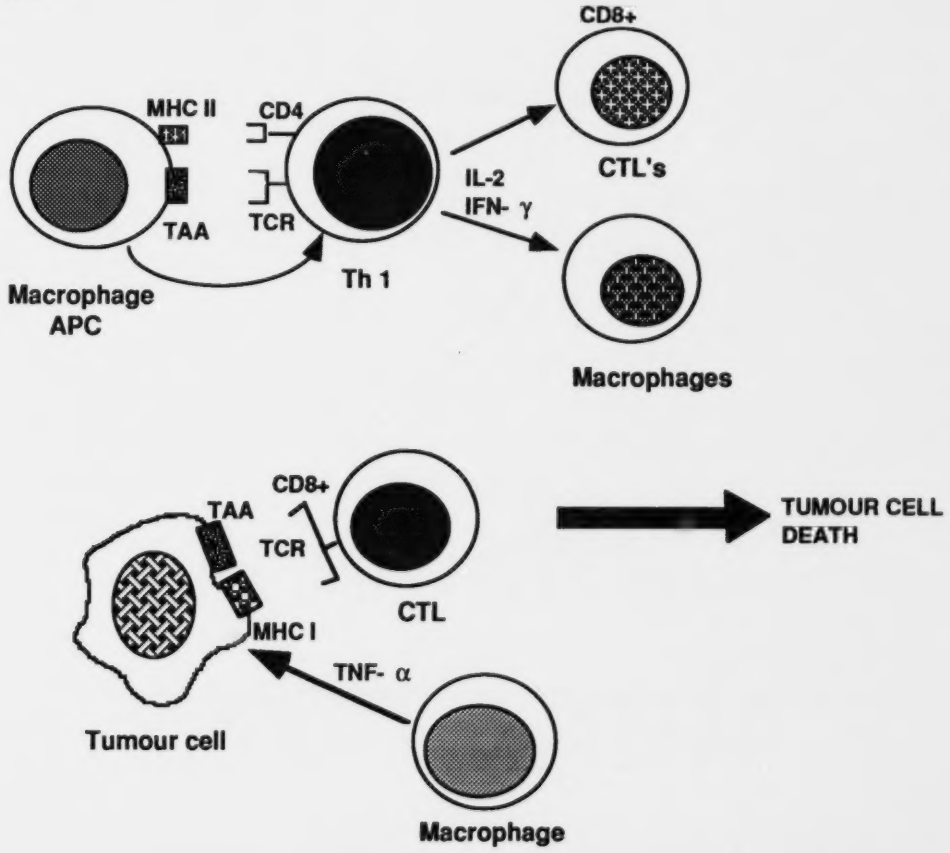
GBM cells express a MAGE-1 encoded protein(111) which has been shown to be antigenic in melanoma cells via specific recognition by T-cells(112).

Cell-mediated immune responses

There are 3 arms to cell-mediated immunity

(i) Ab-dependent cell-mediated immunity. This occurs to a minimal extent in gliomas

(ii) Ag-specific MHC restricted T-cell receptor mediated cytotoxicity. This process is shown in diagrammatic form below. The activated T-lymphocytes in conjunction with TNF-α secreted by activated macrophages normally results in tumor cell death.



The figure above shows the various processes involved in cell-mediated immune responses.The full form of the abbreviations is to be found in the accompanying text.

In patients with GBM's, however these mechanisms are defective. The relative lack of Th cells in the TIL's of gliomas results in lack of stimulation by the TAA resulting

in T-cell anergy and failure to produce activated cytotoxic T-lymphocytes (CTL's)(113, 114).

In vivo, glioma cells have been shown to have low levels of expression of MHC class I molecules(115-117).Thus even if CTL's are produced ,the lack of MHC I molecules on tumor cells results in lack of recognition and binding of the tumor cell with subsequent lack of cytotoxicity(118). TGF-β2 secreted by glioma cells may result in MHC class I down-regulation.

(iii) Non-MHC restricted killing of tumor cells by natural killer(NK) cells ($CD2^+CD3^-$)which recognize these cells through NK receptors. Few NK cells are found in gliomas (119); glioma cells are also resistant to NK mediated cytolysis(120).

Non-MHC restricted cytotoxicity can also be mediated by CTL's($CD2^+CD3^+$) which form the basis for lymphokine activated(LAK) cell cytotoxicity. These cells are not normally present in gliomas.

Antigen presentation

Antigen presentation on MHC II molecules by macrophages with recognition by $CD4^+$ Th cells is the first step in the recognition of foreign Ag's. Astrocytes, endothelial cells, B-cells and some T-lymphocytes can act as antigen presenting cells(APC's) in the CNS. Even glioma cells can express MHC class II molecules and thus act as APC's. However, only macrophages are professional APC's because Ag presentation is a specialized process involving two signaling steps.

Signal 1 involves the ligation of the T-cell receptor(TCR) by specific peptide MHC complexes which can also be done by non-professional APC's.

However, signal 2, which is antigen non-specific is carried out by the B7 molecule which is exclusively expressed on professional APC's. The B7 molecule interacts with the CD28(CTLA4) receptor expressed on T-cells. If signal 2 is not present the Th cell goes into a state of inactivation or T-cell anergy(121, 122).These $CD4^+$ cells(cytolytic T-cells) kill colleague T-cells through the induction of apoptosis resulting in the depletion of effector Th cells.

It has been shown that high levels expression of MHC class I molecules correlates with a good prognosis while high levels of expression of MHC class II molecules correlates with a poorer prognosis in patients with GBM's(123, 124).

Immunomodulatory molecules produced by glioma cells

TGF-β

TGF-β is a large family of polypeptides with a vast range of target cells. TGF-β2 is the major isoform detected in patients with GBM's. Normal astrocytes secrete both TGF-β1 and TGF-β2 in a latent non-active form containing an extra-peptide sequence(latent TGF-β binding protein LTBP).This has to be proteolytically cleaved to the active form.

Glioblastomas secrete both the latent and active forms and also a factor which activates latent TGF-β1 from human platelets(125).TGF-βs have numerous immune-modulatory functions which may play a role in the immunesuppression seen in patients with GBM's. They also induce many components of the extracellular matrix including collagen, fibronectin and cell surface integrins which may promote tumor growth(107). At the same time the TGF-β's decrease the synthesis of enzymes such as collagenase and transin/stromelysin that help break down such matrix components.

They also increase the levels of inhibitors of these enzymes including plasminogen-activator inhibitor 1. They promote angiogenesis by increasing extracellular matrix formation and by a direct mitogenic effect on endothelial cells.

The receptors expressed on glioma cells may be functionally different from those on normal astrocytes as TGF-β enhances the growth of glioblastoma cells whereas it inhibits the growth of normal astrocytes(126, 127).TGF-α interacts with RB1 tumor suppressor gene inactivating it; this may promote glioma genesis(128).

IL-10

IL-10 is a cytokine produced by Th cells which act on Th1 cells to inhibit their ability to produce other cytokines resulting in a shift of the immune response to one of Ab production rather than cell mediated reactions. It acts on macrophages to inhibit the expression of MHC class II and cytokine production mostly IL-1 and TNF resulting in the inhibition of antigen-specific T-cell proliferation. It concurrently enhances the viability and MHC II expression of B-cells resulting in enhancement of Ab production. IL-10 probably acts as an immunesuppressive factor in glioblastomas. It further stimulates glioblastoma cells to increase their production of IL-β1, IL-6 and GM-CSF.

Prostaglandin E_2

Glioblastoma cells secrete a significant amount of prostaglandin E_2 both *in vitro* and *in vivo* (129). PGE_2 profoundly suppresses the production of IL-2 and IFN-γ by the

Th1 subset and T-cell expression of IL-2 receptors. This again favors Ab production rather than cell-mediated reactions. In gliomas, PGE_2 together with TGF-β2 and IL-10 synergistically suppress cellular immunity.

Tumor Necrosis Factor-α (TNF)

Macrophages or microglial cells are the main source of TNF in the CNS. Normal astrocytes will produce TNF upon stimulation by IL-1(130, 131). *In vivo* expression of TNF by glioma cells has not been seen thus far though they express receptors for TNF. Although most glioma cells are resistant to the cytotoxic effect of TNF, it can still cause G_1 arrest of proliferation and increase the production of a number of cytokines and cell surface molecules(132).There are two types of TNF receptor, p55 and p75. Glioma cells predominantly express the p55 receptor which transduces most of the functions of TNF-α.

IL-1

IL-1 plays a key role in inflammatory and immune responses. There are 3 different types, IL-α, IL-β and IL-1 receptor antagonist(IL-1ra)(133). IL-α but not IL-β has been found in GBM's in culture(134). IL-1 acts on glioma cells to produce various effects including growth promotion, up-regulation of inter-cellular adhesion molecules, down-regulation of MHC class II, and increase in cytokine production. There are 2 IL-1 receptors, p80 IL-1R and p68 IL-1R. GBM cells predominantly express the former which has a greater affinity for IL-1α than for IL-1β. The overall result would be one of stimulation of glioma growth.

Interferons

IFN-α/β bioactivity has been reported in GBM culture supernatants(135) though their production by GBM cells seem doubtful as they frequently lack the genes for IFN-α and -β(136, 137).The detected activity may been due to IL-6 which is functionally similar and used to be called IFN-β2. Thus far IFNγ has not been seen in gliomas. IFN genes act as tumor suppressor genes; transfection of GBM cells with the IFN-β gene causes growth inhibition(77). Inhibition of IFN-β gene expression results in de-repression of *c-fos* (138) and suppression of MHC I expression. c-*fos* acts as a promoter of various cytokine genes including those for IL-1α, IL-6, IL-8 and TGF-β. Thus IFN gene deletion might be the cause of increased cytokine production by GBM cells. Glioma cells express both IFN-α/β receptors and IFN-γ receptors. IFN-β acts via these receptors to cause suppression of MHC class II expression on glioma cells while IFN-α and -γ cause growth

inhibition. The latter also causes ICAM-1 induction, MHC class II induction and growth modulation.

Interleukin-6

IL-6 is produced by GBM cells(139) as a result of either IL-1 stimulation or de-repression of the IL-6 gene by the p53 gene product(140).The biological role of this cytokine has not been elucidated though it does play a role in angiogenesis(141).

Colony stimulating factors

Granulocyte-macrophage colony-stimulating factor(GM-CSF) is produced by glioma cells *in vitro* but not *in vivo* (134). Its absence in glioma tissue may be due to suppression of GM-CSF production by TGF-β2 and PGE_2. Granulocyte CSF(G-CSF) has also been found in glioma cells in culture(142).

Macrophage CSF(M-CSF) is found in the CSF of patients with cranio-pharyngiomas, meningiomas and to a lesser extent in astrocytomas. The majority of glioma cells express the M-CSF receptor(M-CSFR)gene. M-CSFR is identical to the *c-fms* proto-oncogene product and is a tyrosine kinase receptor. It may therefore play a role in the growth and progression of malignant gliomas.

The stem cell factor receptor (SCFR),which is the *c-kit* proto-oncogene product and a tyrosine kinase receptor is uniformly expressed by GBM cells(143).

Interleukin-8

IL-8 is produced by glioma cells both *in vitro* and *in vivo* (144)and is also found in the CSF of patients with GBM's. IL-8 is a potent chemotactic agent for T-cells and neutrophils and may be responsible the T-cell infiltration seen in GBM's rather than the latter being due to specific TAA recognition. IL-8 is also a potent angiogenic factor and is produced by endothelial cells thus setting up an autocrine and/or paracrine growth stimulatory loop.

MCP-1

Monocyte chemoattractant protein-1(MCP-1) is a cytokine that induces selective chemotaxis of macrophages. Some GBM cell lines produce this factor (145, 146)which may be responsible for the macrophage infiltration seen in glioma tissues.

The functional role of tumor infiltrating macrophages is unclear. If fully activated they could kill tumor cells or initiate tumor cell rejection through their function as APC's.

However they may also act to stimulate neoplastic proliferation through either growth factor production or induction of neovascularization(147).

It can thus be seen that there is a major derangement of the immune system in patients with GBM's. This is mostly the result of various factors secreted or conversely not secreted by glioblastoma cells. The immune system may thus be boosted or augmented by inhibiting the action of some of these factors(TGF-β2) or by addition of the missing factors or genes. Gene therapy could therefore play an important role in helping to augment the immune responses in GBM patients which in turn could lead to rejection of the tumor or, at least, inhibition of growth and or further progression of the tumor.

Treatment of brain tumors

The treatment of brain tumors may be divided into surgical and non-surgical. The current therapy of brain tumors involves various combinations of surgical and non-surgical treatment modalities

Surgical Treatment

Neurosurgical treatment is required for diagnosis, decompression, cytoreduction and when possible, cure.

Tissue samples are needed to confirm both the nature/histology of the tumor and its grade. Bromodeoxyuridine labeling of tissue samples gives a fairly accurate indication of the labeling index and thus tumor aggressiveness(38, 39).

Decompression of the tumor is needed when by sheer size, swelling or hydrocephalus, it has produced a significant neurological deficit. The goals of surgical treatment are to lower intracranial pressure, reverse patients symptoms and prevent the onset of new deficits. Surgical decompression can extend the life span of a patient and in a emergent situation can save lives. This is particularly true of tumors located in the temporal lobe and posterior fossa where they can cause uncal and tonsillar herniation respectively.

A number of studies (not all adequately designed) have shown a correlation between cytoreduction and length of survival in patient with astrocytic tumors. Cytoreduction reduces the tumor burden but also facilitates adjuvant therapies. This may be due to a decrease in the number of tumor cells, altered cell kinetics, removal of radio-resistant hypoxic cells and / or removal of areas of the tumor inaccessible to chemotherapy(148-153).

In certain types of tumor, long disease free intervals and sometimes cure can be accomplished by removal of the tumor. This is also the case in patients with solitary brain metastases where surgery and radiation resulted in better survival than radiation alone (40 vs 15 weeks)(154).

Resectability of a tumor is determined by the following factors:

(i) The location of the tumor; those in eloquent areas such as the brainstem, hypothalamus, etc. may not be completely resectable.

(ii) The tumor's size and multiplicity.

(iii) The nature of the tumor margin, i.e. infiltrating vs well circumscribed (encapsulated)

(iv) Pre-operative neurological status depends on whether the deficit is due to tumor bulk and swelling or to tumor infiltration; the former is treatable.

(v) The patient's overall medical condition and age.

In the case of gliomas, especially malignant gliomas an attempt is made at surgical debulking if possible given the above constraints.

Re-operation for a recurrent tumor has a narrower risk : benefit ratio compared to newly diagnosed tumor. It is needed for :

(a) tissue diagnosis as tumor may have changed in grade (anaplastic to glioblastoma or undergone sarcomatous changes. It also helps to distinguish radionecrosis from tumor.

(b) Surgical decompression may relieve symptoms due to tumor mass or swelling.

(c) If a seizure disorder is worse due to tumor regrowth, surgery may help reduce the seizure frequency.

(d) Cytoreduction in order to facilitate adjuvant therapies.

The patient's pre-operative neurosurgical status(often graded on the Karnofsky scale) is one of the most deciding factors in the decision to re-operate.

The interval to recurrence of the tumor will also play a role as patients who present in less than six months since the first operation do poorly.

Stereotactic Surgery

Stereotactic surgery is an alternate form of surgery for certain kinds of tumor (i.e. deep seated virtually non-resectable lesions) such as those in the brain stem . A lesion requiring adjuvant treatment before resection such as a sarcoma, a suspected abscess, small lesions that are difficult to localize by other methods or a patient who is medically unfit to undergo a major craniotomy are all possible indications for using a stereotactic approach.

Radiotherapy

Numerous randomized trials have established the role of external- beam conventional radiation in the post-surgical treatment of patients with malignant gliomas with an improvement in median survival from 14 - 22 weeks to 36 - 47 weeks. However, local recurrence of tumor results in most patients dying of recurrent tumor within 2

years. Innovative and experimental radiotherapeutic approaches have been developed in an attempt to better this.

Conventional fractionation

Conventional external beam radiation is usually administered daily over a 5 -6 week period delivering 50 to 60 Gy in 1.8 - 2.0 Gy fractions. Various studies have been done comparing the total radiating dose and survival. A trend towards better survival was seen with increasing doses up to 60 Gy(155). The last study by the MRC compared 45 Gy in 20 fractions to 60 Gy in 30 fractions in 474 patients - two thirds of patients were in the in 60 Gy group(156). Median survival was improved from 9 months to 12 months in the 60 Gy arm: the survival curves however became superimposed beyond the second year. Previously the whole brain was irradiated, but in the last 10 years, based on studies that showed most recurrences occurred within 2 cm of the initial tumor volume , radiation is delivered in regional fields encompassing the tumor with a 2-3 cm margin as the initial volume with a boost in a smaller volume for a total dose of 50 -60 Gy(157, 158).

Altered fractionation

Accelerated fractionation delivers the same total dose as conventional fractions over a shorter period of time using 2 to 3 daily fractions of conventional size. In rapidly dividing tumors such as glioblastomas, the goal is to reduce overall treatment time and reduce the possibility of tumor cells repopulating during treatment. Each daily fraction is separated by an interval of 4 hours or greater to allow some repair of sublethal damage inflicted on rapidly dividing target cells such as scalp epithelium(159, 160).

Two small studies using 40 Gy in 20 fractions in 7 days and 60 Gy in 1.6 Gy fractions 3 times a day over 16 days showed no improvement in survival but also no increase in toxicity(160). An RTOG study found that patients greater than 60 years survived longer- 10.4 months at 54.4 Gy when administered as 1.6 Gy fractions twice daily. There was no increase in toxicity(161).

The EORTC used 3 fractions of 2 Gy / day with a 4 hour gap to deliver 30 Gy in 1 week. This was repeated after a 2 week break for a total of 60 Gy in 30 fractions in 4 weeks. There was no change in median survival(162).Thus accelerated fractionation has not resulted in improved survival but these shorter regimens have been well tolerated with no increase in late sequelae.

Hyperfraction delivers a total dose 10 - 20 % greater than conventional RTX over the same period using 2 to 4 daily fractions that are smaller than conventional fractions. Normal glial and vascular cells limit the total amount of RTX that can be administered . These cells divide slowly and are better able to repair sublethal damage compared to neoplastic cells. Using the above technique, improved repair of sublethal damage at lower sized fractions might allow a higher total dose to be associated with the same degree of late sequelae. Since neoplastic cells divide relatively rapidly, the increased number of daily fractions would enhance the chance of radiating them at a more sensitive phase of their cell cycle. Also at smaller radiation doses, cell killing is less dependent on oxygen which would be useful as hypoxic areas are common in tumors. Numerous studies have been done to assess the efficacy of hyperfractionation. Fulton et al showed no improvement when the total hyperfractionated dose was increased from 61.4 to 71.2 to 80 Gy. (Median survival of 46, 30, and 45 weeks)(163). A BTSG study showed no change(164). An RTOG study showed equivocal results and a study is currently underway by them comparing hyperfractionated RTX 72 Gy vs 60 Gy conventional (161) .

Radiosensitizers

These are chemicals that increases the lethal effects of RTX. There are 2 major classes:

Hypoxic cell sensitizers - Lab studies have shown that hypoxic cells are 2.5 - 3x more resistant to treatment than well oxygenated cells. Hypoxic cell sensitizers would sensitize the hypoxic tumor cells without increasing the RTX effects on the already well oxygenated normal tissue. Nitroimidazoles are electron-affinic radiosensitizers that enhance RTX effectiveness by 1.3 to 1.7 X. They reach peak serum levels within 2 to 3 hrs and equilibrate with the CNS over 4-6 hrs. The principal side effects are peripheral neuropathy, gastro-intestinal upset and occasional CNS toxicity manifested by convulsions and psychosis.

Urtasun et al(165) initially reported on the effect of metronidazole in a small, randomized study in 1976. Since then 6 randomized studies have not shown any benefit from the addition of misonidazole to various combinations of RTX and chemotherapy (164, 166-170).

The intra-tumoral concentration of misonidazole may not have been adequate as a result of the above neurotoxicity. Re-oxygenation may also occur during the 5-6 weeks of radiation treatment to counter the effects of hypoxia.

Studies are currently underway using the next generation of sensitizers (pimonidazole [Ro 03-8299] and etanidazole[SR 2508]) that are more potent and achieve higher intratumoral concentrations because of their differing toxicity profile(171, 172).

Hyperbaric oxygen has also been tried for the same reasons with minimal improvement in survival.

Halogenated pyrimidines

BrdU and IUdR are thymidine analogues with halogens being substituted for a methyl group, and as such, are incorporated into DNA in place of thymidine in a competitive manner. Mitotically active tumor cells are thus more likely to incorporate these compounds than the slowly replicating vascular cells and glial cells in normal brain.

Both intra arterial and intravenous routes have been tried. The former is more attractive as these compounds are dehalogenated by the liver and thus this route would deliver larger concentrations to the CNS and tumor(173-175) .

Greenberg et al using a special indwelling intra-arterial catheter delivered bromodeoxyuridine over a several weeks course of radiation treatment with a median survival of 22 weeks in 18 patients(176).

A group at the National Cancer Institute found no change in survival using the intravenous route(177). This may have been due to limited access across the blood-brain barrier, a high intrinsic cellular radioresistance and zones of tumor with a poor blood supply. A study at UCSF found an increase in median survival from 82 to 252 weeks in points with anaplastic astrocytomas but none in glioblastomas(178).

A study is now currently underway comparing 60 Gy in 30 fractions sensitized with hydroxyurea vs 60 Gy BrdU - both groups being followed by PVC chemotherapy (179).

Interstitial Brachytherapy

Interstitial brachytherapy involves the stereotaxic implantation of radioactive seeds (I^{125} or Ir^{192} or Au^{98}) interstitially in tumors. The rapid decrease in dose outside the high-dose volume results in minimal dose being delivered to the surrounding brain and a higher dose to be delivered to the tumor.

Certain criteria should be met for brachytherapy: Tumors should be no larger than 5-6 cm and supratentorial (preferably peripheral) without any involvement of the corpus callosum). Patients should have a Karnofsky of greater or equal to 70. Interstitial brachytherapy is often used for patients with recurrent tumors. Leibel et al reported on 98 patients with recurrent malignant gliomas treated with high-activity I^{125}. Patients also

received external beam radiotherapy and most had received chemotherapy. Patients with recurrent glioblastomas had a median survival of 54 weeks(180). Up to 12000 rads may be administered . 25-50% of patients however require re-operation because of resulting radiation necrosis causing mass effect. Most other points are steroid dependent.

49 patients with recurrent gliomas were treated with brachytherapy and interstitial hyperthermia at UCSF. These patients had a treatment immediately before and after the loading and removal respectively of the catheters. Outer catheter were initially placed with dummy sources to verify their position. The latter were replaced with 915 MHz helical coil microwave antennae. Multisensor fiber optic thermometry probes were placed into separate thermometry catheters and the coils heated to achieve a temperature of 43.2C for 30 minutes in as much of the treatment volume as possible. Patients with recurrent GBM had a median survival of 47 weeks (45% 1-year survival) while those with recurrences following an initial diagnosis of anaplastic astrocytoma had a 1 year survival of 81% with a median survival that has not been reached(181-183).

Numerous studies have shown some benefit in recurrent tumor cases. 88 vs 67 (UCSF) for glioblastoma(184). Loeffler's group, using a historic control group, did find improved survival for both anaplastic astrocytomas and glioblastomas(27 months median survival), but the groups differed in other characteristics(185). 2 randomized studies are currently underway (BTCG and University of Toronto) comparing external beam radiation and interstitial implant boost and chemotherapy vs external beam radiation and chemotherapy. Glioblastoma patients seem to benefit more than those with anaplastic astrocytomas.

Particle Therapy

Particle therapy uses subatomic particles instead of photons. These include neutrons, protons, helium and Neon nuclei and negative pi mesons (pions). These particle beams offer better dose localization to the tumor volume and greater biologic effect compared to photons.

Different neutron boost schedules including the use of **fast neutrons** generated by a cyclotron found no difference in survival ; lower neutron boosts caused recurrent tumor, higher boosts greater radiation necrosis, i.e., no safe therapeutic window is available to date.

Boron-neutron capture therapy involves the administration of boron-containing compounds which are selectively taken up by tumor cells and subsequently treated with slow or thermal neutrons. This results in the generation of Li and He fragments which damage the individual cell as shown:

$^{10}B + {}^{1}n = (^{11}B) = {}^{7}Li + {}^{4}He = 2.4MeV$

This has not shown promising results mainly because of poor localization of boronated compounds purely in tumor cells and not in other cells especially vascular cells.

Pions are produced by a large high intensity, high energy proton accelerator. They have 1/6 the mass of a proton and 273 x that of an electron. They possess both a physical dose localization advantage (Bragg peak) and an increasing biologic effect. Only 2 facilities are available worldwide (Vancouver and Zurich). Studies are currently underway to test this treatment modality.

Proton beam therapy has advantages in that the high dose volume can be restricted to the tumor because of the Bragg peak effect. Biologically, protons have 1.10 x the biologic effect of photons.

A group in Boston have shown improved survival in points with skull base chordomas and low grade chondrosarcomas. A study using 90 CGy is under way to treat malignant astrocytomas.

Hyperthermia

Heat is cytotoxic as a single modality. Cells in S-phase (more resistant to X-rays) are sensitive to heat; cells in a low pH and hypoxic environment (resistant to X-rays) are more sensitive to heat; heat inhibits the repair of sublethal damage from X-rays - it has a more than additive affect when combined with X-rays. It also augments the effect of some chemotherapeutic agents.

Various methods are used to generate heat ; magnetic loop induction, interstitial microwave antennas and radio frequency. A major limitation is the inhomogeneity of tumor volume heating.

Interstitial microwave antennas used concurrently with interstitial therapy have been tried with some effect (183).

Other studies are also underway (phase II) comparing external beam and hydroxyurea followed by 1^{125} implant with and without hyperthermia(186)

Chemotherapy

All chemotherapeutic agents work by affecting DNA synthesis or function and usually do not kill resting cells. Most of these agents act at specific phases of the cell cycle and are most effective in killing dividing cells. The BTSG initially showed that 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) did produce a modest improvement in survival; from 9 to 12 months to 12 to 18 months in patients with malignant astrocytomas(187). Other drugs used include CCNU, PCNU, ACNU, procarbazine , hydroxyurea, dianhydrogalactol, etoposide, VM-28, bleomycin and cisplatin. The 3 drug PVC (procarbazine, CCNU and vincristine) combination has been shown to have a slight advantage over carmustine in patients with glioblastomas and anaplastic astrocytomas(188).This is particularly true in anaplastic astrocytomas: 157 weeks vs 82 weeks(189).

Intracarotid chemotherapy results in the regional delivery of high concentrations of drug to the tumor with less reaching the systemic circulation. However various studies have shown only marginal benefit. The biggest drawback is the high incidence of neuro-toxicity and especially retinal toxicity (190)(the latter can be decreased by using a supra-ophthalmic artery-sited catheter). Intracarotid cisplatin and methotrexate have been used in this way.

Aggressive chemotherapy with super high doses of chemotherapy followed by bone marrow transplantation has been tried using carmustine alone or in combination with AZQ (a semisynthetic azoquinone) or etoposide. These treatments have been associated with high levels of toxicity(191, 192)(opportunistic infections, veno-occlusive disease of the liver, graft vs host disease and complications from immunological incompetence.)

Various drugs and combinations have been tried for recurrent malignant gliomas. Procarbazine as well as AZQ with and without carmustine have shown some benefit(193, 194).

DFMO has been shown by Levin et al (195)to be effective against slower growing anaplastic astrocytes when used in combination with mitoguazone; 46% response with MTP(mean time to tumor progression) of 49 weeks. Only 17 % of glioblastomas responded(195).

Blood-Brain Barrier Modification and Chemotherapy

Reversible BBB disruption (197, 1978) using intracarotid mannitol has been tried in combination with intra-arterial chemotherapy (methotrexate, cyclophosphamide and oral procarbazine.) This results in a larger concentration of agent reaching the tumor(199). However, tumor responses on the side of the infusion were occasionally accompanied by the development of new lesions in other regions of BBB disruption. Neurological complications include transient exacerbations of neurological deficits and a 15% incidence of seizures. One of the problems with this treatment is that BBB disruption also occurs in normal brain resulting in high concentrations of drug in normal tissue.

Other methods of chemotherapy delivery

Various other carrier agents have been tried including the *dihydropyridine - pyridium salt redox system*. This lipid soluble compound crosses the BBB. Biological oxidation results in the quarternary ester which is ionic and is locked in the BBB(200-202).

Topical chemotherapy- in order to produce high concentration of drug locally various topical methods have been tried including

a) use of implanted catheters (203)

b) negatively charged liposomes carrying chemotherapeutic agents(204-206)

c) use of biodegradable wafers (Biodel polymer impregnated with chemotherapeutic agents). Studies have been done using BCNU with a slight improvement in survival with minimal systemic toxicity(207-209).

Drug Resistance of Tumor Cells

One of the problems with chemotherapy is the development of drug resistance of the remaining tumor cells which are selected out and become the dominant cell population thus rendering treatment for recurrent tumors more difficult.

There are various mechanisms of drug resistance including the presence of **enzymes** to repair DNA damage produced by various chemotherapeutic agents (for example methyl reductase)(210).

Pleotropic drug resistance where by chemotherapy resistance may be transferred to previously sensitive cell lines through a gene-transmitted membrane effect(211).

The **multi drug resistant gene** produces resistance to a wide variety of other anti-neoplastic agents following treatment with a particular class of agent. This may be modified to an extent using cyclosporin A or FP318(211-214).

The P-glycoprotein molecular pump ejects certain drugs for example, anthracyclines (vinca alkaloids). Radiotherapy increases the proportions of cells producing P-glycoprotein. Calcium channel blockers such as verapamil block this pump and may be used in conjunction with this class of chemotherapeutic agents.

Photoradiation Therapy

Certain substances (photo-sensitizers) preferentially concentrate in malignant tissue. These substances selectively destroy tissue when activated by light of a certain wavelength and intensity in the presence of oxygen. Electrons are promoted by photon absorption to an excited triplet state which then interact either directly with substrates within the cell or indirectly with the substrates through the production of singlet oxygen. This results in damage to cell membranes, cytoplasmic organelles (lysosomes), nuclear DNA, etc. culminating in cell death.

The most widely used is HPD (215, 216), a derivative of hematoporphyrin which absorbs a range of wavelengths; the most commonly used clinically is 624 nm or red light as it penetrates tissue better. The light is delivered in one of three ways ;

1) Stereotactic implantation of one or more quartz fibers to provide argon dye laser photoradiation.

2) Topical delivery system consisting of a filtered high intensity xenon-arc lamp and a fibreoptic cable with a lucite tip inserted in a diffusion medium which fills the tumor bed.

3) Cystic-cavitary lesions may be filled with the diffusion medium and illuminated with either a laser-quartz fiber system or high intensity xenon-arc lamp fibreoptic system.

The results of various studies to date(217, 218) have not been encouraging in terms of prolonging median survival. This is probably due to the nature of the tumor and the limited penetration depth of this technique.

Immunotherapy for human gliomas

There is a marked generalized depression of immunecompetence affecting both cellular and humoral mechanisms in patients with gliomas. Delayed hypersensitivity reactions to various test antigens are depressed; the degree of cellular anergy parallels progression of the tumor(219). There is a general decrease of T-cell numbers(220-222) with decreased production of IL-2 and /or expression of the IL-2 receptor by stimulated lymphocytes(223, 224). Transforming growth factor beta (TGF- β) is secreted by glioblastomas(225, 226) and inhibits T-cell proliferation and generation of immune

responses by interfering with IL-2 dependent pathways. Patients with gliomas also have an impaired ability to generate antibodies to new antigens. Immunoglobulin levels are normal except for increased levels of IgM.

Some cytotoxic antibodies against certain glioma associated antigens have been found. However, it remains controversial whether they alter the prognosis in any way(227).

Various types of immunotherapy have been tried in glioma patients, usually with little or no beneficial effect.

1) **Active specific immunity**- by injecting patients subcutaneously with autologous tumor cells(228-230).

2) **Active non-specific immunotherapy**- this method attempts to increase the general immunecompetence of the host. This includes using purified protein derivative (PPD) injections as well as Levamisole. Mahaley et al found no benefit in patients using the latter technique(231).

3) **Adoptive immunotherapy**- this technique attempts to confer immuno-competence to the host by the transfer of cellular elements of the immune system. Results have been inconclusive(232).

Lymphokine Activated Killer Cells

Lymphokine activated killer cells have been utilized to this end by injecting autologous LAK cells and/ or interleukin-2 (IL-2) into the cerebral tissue surrounding the cavity following brain tumor resection. Lymphokine activated killer cells have been shown to possess tumoricidal activity against a variety of human tumors both *in vitro* and *in vivo*. They produce a broad spectrum of non-MHC restricted oncolysis when activated by interleukin-2. Human trials are currently under way to try and duplicate the preliminary results reported against metastatic melanoma and renal cell carcinoma(233, 234).

Recent modifications in the delivery of systemic LAK/interleukin-2 have substantially reduced the marked toxicity seen with this treatment. LAK mediated lysis of fresh glioma cells has been seen in vitro(235). No significant toxicity was seen following the injection of up to 10^{10} LAK and 10^{6} Cetus units of interleukin-2 into the brain surrounding the tumor cavity(236). Results have been equivocal(237-241).No controlled study has yet demonstrated actual clinical benefit though occasional patients may survive longer. The applicability of LAK therapy for brain tumors by intra-lesional injection is limited by the diffusely infiltrative nature of malignant gliomas, the lack of tumor honing capability, the absolute dependence of LAK activity on the continuous presence of exogenously administered interleukin-2, the relative absence of lymphocytic

traffic in the brain and the presence of potent, tumor-secreted inhibitors of LAK activation.

A pilot study was undertaken at the M.D.Andersen Cancer Center(242) using interleukin-2/LAK in children with primary brain tumors who had mostly leptomeningeal deposits. The patient's lymphocytes were pretreated with anti-CD3 Ab prior to culturing with interleukin-2 in order to produce sufficient numbers of cells(243).Tumor cells circulating in the CSF could be cleared but solid tumor masses remained unaffected.

Tumor Infiltrating Lymphocytes(TIL)

TIL's from gliomas may be clonally expanded using OKT3 amplification of interleukin-2 mediated proliferation.(244).Animal studies have shown increased efficacy of TIL's over conventional LAK cells(245). Preliminary results from the National Institutes of Health have shown a high rate of tumor response to TIL infusion. The TIL, when optimally activated in vitro, may locally proliferate in situ and recruit other effector cells to maintain the desired tumoricidal effect(246).

Cytotoxic T Lymphocytes(CTL)

MHC-restricted CTL's can be generated by co-culturing the patient's peripheral blood lymphocytes and tumor cells. One clinical trial has been reported involving 5 patients in whom CTL's were administered directly into the intra-cranial tumor cavity(247).The results were encouraging with some tumor responses.

4) **Passive immunotherapy**- this involves injecting antibodies to various glioma associated antigens(including tenascin). These can be tagged to various therapeutic agents.

Radio-immunotherapy involves tagging various radioisotopes to specific antibodies. These include the beta-emitters I^{131}, Y^{90}, Cu^{67}, Re^{176}, and alpha emitters At^{211} and Bi^{212} (248).

Because RIT can kill cells 50 or more cell diameters from the binding site, it has advantages over chemo- immunetherapy. Mixed results have been obtained because of the problem of finding antibodies which will enter a majority of tumor cells to achieve cell kill. Certain TAA characteristics must be met :

1) Level of antigen expression- this may be increased using β- and γ- interferon. RTX and certain chemotherapy agents(249) shift cells into the G2 and M-phases of the cell cycle where TAA are preferentially expressed.

2) Degree of antigen movement

3) Antigen accessibility.

I^{131}-conjugated monoclonal antibodies to glioma-associated antigens have been shown to alter tumor growth in nude mice(250-252).The transferrin receptor, which is a proliferation associated marker, is more uniformly expressed in gliomas and hence would

be a more suitable target for Ab binding. Immunotoxins that couple the transferrin receptor with ricin have potent in vitro tumoricidal activity against gliomas and other tumor cell lines(253).

Effector cell function can be markedly enhanced by antibody targeting(254). Heterobifunctional conjugates offer the possibility of directing specific effector cell populations to the target site(255). Conjugation of an anti-CD3 Ab to an anti-transferrin antibody would direct CD3 bearing lymphocytes to the region of the glioma.

Growth factor antagonists or toxins conjugated to tumor-associated growth factors may offer new approaches for treatment.

Immune-modulation

Interferon

Interferon-β has a growth inhibitory influence on glioma cells. Phase II clinical trials for the treatment of malignant gliomas resulted in a small number of patient responses(a response rate of 51% was seen with recombinant IFN-β with an MTP of 26 weeks) mostly in patients with anaplastic astrocytomas(256).

IFN-γ has many immune-modulatory functions including the induction of class I and II major histocompatibility complex(MHC) molecule expression. Neoplastic astrocytes have demonstrated class II antigen expression(118, 257). Glioma cells will express class II antigens when exposed to IFN-γ. The amplified expression of MHC class II molecules in the region of the brain tumor may enhance the homing ability of adoptively transferred cytotoxic T lymphocytes(CTL's) and TIL's.

Interleukin-1 and TNF

Interleukin-1 functions as an endogenous adjuvant during lymphocyte activation and is a primary mediator of the acute-phase inflammatory response. TNFα shares many of these properties and the two may act synergistically in inflammatory reactions and tumor necrosis(258). In vitro expanded lymphocytes which have been pretreated with these cytokines show increased oncolytic activity(259). Cytokine-induced immune-modulation may thus be necessary prior to the intracavitary infusion of TIL, CTL or LAK to achieve maximum tumoricidal effect.

Interleukin-2

Interleukin-2 plays an obligatory role in LAK cell activation and proliferation (260, 261). LAK cells can recognize and lyse tumor cells in a non-MHC restricted manner. Various methods of interleukin-2 delivery ,when used alone, have been

explored(245, 262). Intravenous injections cause a characteristic toxicity which may be due to a vascular leak syndrome(263), as well, blood-brain barrier dysfunction has been seen. Intraventricular administration has been tried in patients with metastatic leptomeningeal tumor deposits(263).Transient fever and meningeal irritation was seen. In some patients this agent caused an increase in intracranial pressure(nausea, vomiting and/or stupor) necessitating a reduction in dose. Clearance of tumor cells from the CSF did occur but effective therapy could only be maintained with regular infusions. TNF-α levels were seen to peak 2 to 3 hours following the intraventricular interleukin-2. Lymphocyte migration into the tumor was observed. The cellular response could only be observed within the first few millimeters of brain parenchyma adjacent to the ventricle which suggests that the effect of the interleukin-2 was limited to the diffusion distance of the interleukin-2 or some other secondary cytokine(perhaps TNF-α).

Gene Therapy

Gene therapy is a novel form of therapy that aims to correct various genetically mediated problems caused by a lack of a particular gene or by excess expression of a gene.

There are various ways in which gene therapy can be useful. An unsuccessful attempt was made in 1980 to carry out gene therapy for beta-thalassemia with the use of calcium phosphate mediated gene transfer. Retroviral mediated gene transfer was developed in the early 1980's in animal models. This is the most common method in current use. In 1989 the first federally approved human gene transfer experiment was initiated. It involved the transfer of gene-marked immune cells (tumor infiltrating lymphocytes) into patients with advanced cancer. The study had two main objectives:

1) To demonstrate that an exogenous gene could be safely transferred into a patient and

2) To demonstrate that the gene could be detected in cells from the patients.

This method utilized TILs from patients with malignant melanoma which were transduced with a retrovirus carrying the NeoR gene (from E.coli). TIL-marked and unmarked cells from the patient were reinfused back into the patient. Subsequent sampling showed that gene-marked TILs could be detected consistently in the blood stream for three weeks. TIL infusions were done in conjunction with IL-2 infusion which caused TIL cell proliferation and activation. This study did meet both objectives as outlined above. Advances in recombinant DNA technology have made the prospect of gene therapy to introduce healthy gene into patients to cure disease, a reality.

Genes can be transferred either into germ cells (sperm, eggs or early embryos) or somatic cells. The former is not feasible at the moment and thus somatic cell therapy is the mode of choice. In an ideal situation insertion of a single gene would cure the disease by being site-specific(homologous recombination); the healthy gene would exactly replace the damaged copy.

Targeted insertion is important because it increases the probability that a therapeutic gene will function correctly and decrease the chances of oncogene activation by random insertion.

In reality for every gene spliced into the correct location more than 1000 fit randomly into the genome. Non-targeted delivery of genes into cells can be achieved by either chemical or physical means(transfection) or by viruses (transduction).

In the chemical method, many copies of the gene carrying DNA is mixed with a charged substance- usually calcium phosphate or dextran.

These compounds disturb the cell membrane and result in transport of the gene into the cell. One draw back is that the efficiency of gene integration is so minuscule as to render therapy ineffective.

Physical methods include microinjection with a glass pipette and electroporation (exposure of cells to an electric shock). Microinjection, though efficient in transduction , is too labor intensive to be useful clinically.

Antisense oligonucleotides

Antisense oligonucleotide therapy has been primarily used to prevent activation of a dominant oncogene. Studies have been carried out using antisense RNA strands to the *k-ras* oncogene. The *k-ras* oncogene is found to be mutated in 30-50% of adenocarcinomas of the lung (non-small cell lung carcinoma).The *k-ras* oncogene product, the p21 protein is found to play an important role in signal transduction pathways in tumorigenesis. A plasmid clone was constructed using a normal 2 kb *k-ras* genomic DNA segment carrying 2nd and 3rd exons with flanking intra sequences, subcloned into an Apr-1-neo expression vector in antisense orientation(264).

Cells expressing antisense RNA showed complete inhibition of *k-ras* mRNA synthesis. There was a 3 fold reduction in growth rate in antisense transfectants compared with controls. This was the first study to show that tumorigenecity of a human tumor cell line could be inhibited by antisense RNA to the transforming oncogene.

In human gliomas the EGF-R gene as well as those for PDGF-R and VEGF are overexpressed. This occurs later in the chain of events leading up to a malignant glioma. Its possible that the use of antisense oligonucleotides to the genes coding for these

products may prevent progression of a low grade glioma to a more malignant one or result in regression or infarction of tumor.

Viral Vectors in Gene Transfer

This form of gene therapy utilizes the native ability of viruses to infect cells with resultant integration and expression of the altered genome. Viruses can be grouped as DNA or RNA viruses.

DNA viruses have not been very successful because some do not allow incorporation of further genetic material into their genome whereas others have not been suitable for other reasons. The genetic materials from DNA viruses often stay episomal rather than being integrated into the host cell genome. This decreases the rate and time of gene expression.

Thus most of the focus has been on a group of **RNA viruses**, the retroviruses, whose genome is in the form of RNA. Following infection of the cell an enzyme, reverse transcriptase, produces a DNA copy of the genome which inserts into the host cell's genome.

Retroviruses do have drawbacks:

1) They only enter actively dividing cells and hence only dividing cells are amenable to therapy.

2) Retroviruses, via insertional mutagenesis, can cause cancer if they are allowed to replicate and spread. One technique to overcome the latter, is the production of viruses which lack the genes coding for various proteins important in packaging of viruses prior to their exit from cells. The therapeutic gene takes the place of the missing gene. Thus these viruses are replication deficient and hence at less risk of causing cancer.

Cancer gene therapy

The first cancer gene therapy protocol used the gene for **tumor necrosis factor.** It was incorporated via a viral vector into TILs which were subsequently infused into the patients(244). The TIL homes in on tumor deposits thus subjecting tumor cells to high concentration of TNF which would otherwise be too toxic if given systemically.

Phase I toxicity studies have shown no side effects from this gene therapy. Tumor vaccine type studies have been started using tumor cells transduced with either TNF or IL-2 which are injected subcutaneously into the patient and subsequently the draining lymph nodes are removed and placed in culture to encourage T-cell growth.

The T-cells are then transfused back into the patient with IL-2. This approach is based on animal studies which have shown that immunization with tumor cells transduced with certain cytokine genes produces systemic antitumor immunity mediated by T-cells(233-236).

In this same line of enhancing tumor immunogenecity, studies have been carried using irradiated tumor cells expressing granulocyte- macrophage colony stimulating factor.

Tumor specific T-cells may be used both in adoptive transfer therapies and as reagents for the isolation of new tumor associated antigens. Where tumor associated antigens are expressed by a tumor, antigen presenting cells (for example dendritic cells and macrophages) may be engineered to express a tumor specific antigen in conjunction with specific cytokines to make even more effective vaccines.

Other methods of gene therapy for cancer attempt to introduce tumor supressor genes (such as RB1 or p53) into tumor cells, to render cells resistant to the toxic effects of chemotherapy and to introduce conditionally toxic genes into tumor cells.

Other areas in which gene therapy is currently being applied/ tested is in AIDS, cardiovascular diseases and diseases of the central nervous system.

Viral Mediated Gene Therapy in Brain Tumors

Gene therapy could play a vital role in the treatment and perhaps even cure of brain tumors. This could be achieved in one of three ways;

1) Introduction of tumor supressor genes into the tumor cell in order to restrict growth/ multiplication of the cells.

2) Transfer of genes that encode a particular toxic product which destroys the tumor cells.

3) Introduction of gene whose products specifically induce apoptosis (programmed cell death) in the tumor cells.

In central nervous system tumors, essentially the only cells that are actively proliferating are the tumor cells; the normal neurons and glia are post-mitotically silent and non-replicating. This difference in cell-cycle activity has been utilized in the treatment of brain tumors using viral vectors for the transfer of specific genes.

Herpes virus mutants

Herpes viruses are DNA viruses with a fairly large genome such that other genes can be incorporated into them in order to incorporate these into tumor cells.

One of the problems with the use of the herpes virus is its affinity for neurons with resulting neuropathogenicity. In order to overcome this various mutants of the herpes virus have been investigated as potentially safe vectors in human glioma transduction.

The thymidine kinase gene carried by herpes viruses is essential for their replication. It is expressed by dividing cells but not post-mitotic cells such as neurons and glia in the CNS. Thus mutant herpes viruses(dlsptk) lacking the thymidine kinase gene would be expected to transduce dividing tumor cells sparing normal brain cells and hence minimize any neurotoxicity(265, 266).

Other herpes virus mutants including AraA^{r}9, AraA^{r}13, RE6 and R3616 all show decreased neurovirulence and have been tried in a mouse model of both subcutaneous and intracranial glioma(267).

Herpes virus mutants with mutations in the DNA polymerase gene(268) have been tried as they are replication deficient but carry the thymidine kinase gene which allows control of viral infection using acyclovir. The DNA polymerase mutants prove to be too toxic in vivo causing deaths from early encephalitis. However, these DNA polymerase mutants may be usefully combined with other vectors to create a more effective agent. They have some useful features such as low levels of spontaneous mutation or hypersensitivity to anti-viral agents which may be utilized in the newer vectors.

Another mutant with a mutation in the 34.5 gene also shows attenuated neuropathogenicity(269).These mutants can cause a spreading lytic infection of human U-87 glioma cells in culture. Extended survival with no signs of encephalitis was seen in vivo studies in U-87 gliomas in nude mice even with doses as high as 10^7 particles. Moderate inflammation and some evidence of vasculitis was seen histologically in the absence of clinical encephalitis(267).

However, numerous viral injections have to be made into the tumor area in order to achieve transduction of most of the tumor mass and especially the satellite tumor nodules remote from the parent tumor. The vast majority of malignant gliomas recur within 2 cm of the original tumor due to residual cells at the periphery of the mass. In an attempt to improve the vector bio-distribution problem, replication competent herpes virus mutants are being studied.

Another strategy that has been attempted is the use of tumor cells themselves as producer cells of the virus. This would theoretically allow the virus to spread to remote cells as the producer tumor cells divided and migrated carrying the virus with them. However experimental results utilizing this strategy have been disappointing as the

tumor cells lose many of their malignant phenotypic features including their ability to invade and spread.

Retroviruses

Retroviruses were the first viruses to be utilized and remain the commonest vectors used in gene transfer studies. Retroviruses are RNA viruses which utilize an enzyme, reverse transcriptase to produce a DNA copy of the genome. This DNA becomes incorporated into the host genome thus directing viral protein synthesis. Retroviruses have small genomes thus making stable incorporation of foreign genes for gene therapy difficult.

Retroviruses can be rendered replication deficient by deleting the genes coding for the packaging protein (the psi gene).

Retroviruses are useful in CNS tumors because integration of viral DNA only occurs in dividing cells and hence in the CNS would only enter actively dividing tumor cells and not post-mitotic neurons and glia. Tumors have both proliferating and non-proliferating cell populations and hence multiple treatments (transduction) with the virus may be needed.

In vitro studies have shown effective transductions of cells using the NeoR (neomycin-resistance) gene and using the neomycin analogue G418 to select out cell populations expressing the gene.

However in vivo studies have shown that only 5-10% of tumor cells are transduced under the best of conditions. In order to effect a cure or at least check the growth of the tumor significantly, a larger percentage of tumor cells would have to transduced. The current retroviral vector may be a poor transducing agent because :

1) It is replication deficient and therefore once it enters the cell will not produce further copies of itself which can infect further tumor cells achieving greater tumor kill.

2) Retroviruses only enter actively dividing cells. Since tumors are composed of dividing and non-dividing cells only the former will be transduced.

3) Because of their short half life, they are essentially inactive by the time further cells enter the proliferating pool. In an effort to overcome this problem retroviral vector producer cells(such as NIH 3T3 fibroblasts) have been used.

Foreign genes and promoter elements can be inserted into plasmid DNA equivalents of the retroviral genome which retain the packaging protein gene(psi).These plasmids are transferred into packaging cell lines carrying the wild-type retroviral sequences lacking the psi element required for packaging of their own RNA into virion particles. The packaging cell then inserts the psi-bearing RNA encoded in the foreign gene

bearing retrovirus sequence into virion particles. Only replication deficient viral particles containing the foreign gene of interest are released into the medium. These replication deficient virions can only infect dividing cells resulting in incorporation of the foreign gene into the host cell genome.

Various genes have been inserted into retroviral vectors including the reporter gene lacZ as well as nerve growth factor, tyrosine hydroxylase and onc proteins.

The herpes simplex virus thymidine kinase gene has been incorporated into the genome of a retroviral vector, the Moloney Murine Leukemia Virus (MMLV). This gene product, i.e., thymidine kinase makes the transduced cell sensitive to the anti-viral agents acyclovir and gancyclovir by phosphorylating these drugs which results in inhibition of DNA synthesis.

Both in vitro and in vivo studies have shown that retroviral vectors can be used to introduce HSV.tk gene into tumor cells thus rendering them susceptible to gancyclovir (270, 271).

In vitro studies by Culver et al showed that re-implantation of mixtures of tumor cells(fibrosarcoma cells) with vector producing fibroblasts resulted in a 63 +/- 9 %SEM transduction rate as demonstrated by expression of the NeoR gene(270).

In vivo studies on rat gliomas(C6 cell line) by Short et al have shown modest transduction using a psi-BAG packaging cell line expressing retroviral vectors carrying the lacZ gene(271). The gene product β-galactosidase can be shown to be present by using a histochemical technique using a suitable substrate, X-gal.

Further in vivo studies by Culver et al using fibroblasts engineered to produce retroviral vectors carrying the HSV.tk gene followed by subsequent treatment with gancyclovir produced some unexpected results. Complete tumor regression was seen even though the percentage of transduced cells was much less than 100%. Further studies using various mixtures of tumor cells and tumor cells expressing the HSV.Tk gene confirmed this 'bystander effect'(270).The mechanism of this is not fully understood. Normal cells in the vicinity of the tumor are not affected by this 'bystander effect'. It may be mediated by phosphorylated gancyclovir transferred from the transduced to the non-transduced cells via gap junctions resulting in inhibition of DNA synthesis in these latter cells(see below).

Further studies using a 9L gliosarcoma in Fischer 344 rats showed macroscopic and microscopic tumor regression in 1 of 14 GCV treated rats(272, 273). No toxicity was seen in the surrounding brain. Further toxicity studies by Ram et al(274) in rats and monkeys injected intravenously and intracranially with retrovirus producer cells showed no systemic toxicity. There was some breakdown of the blood-brain barrier locally at the

site of injection causing mild edema and reactive gliosis. This toxic response was eliminated by the use of dexamethasone.

Retroviral producer cells (which produce vectors carrying the HSV tk gene) have also been utilized for the treatment of leptomeningeal neoplasia in Fischer rats(275). The study showed that the producer cells, when given intrathecally, remained viable and transduced leptomeningeal tumor deposits. Subsequent treatment with gancyclovir resulted in a significant prolongation of survival. Injection of producer cells co-infected with replication-competent retroviruses did not result in any further improvement in survival.

An *in vivo* human trial is currently underway at NIH using retroviruses in patients with primary malignant brain and metastatic tumors.

However even with the use of the packaging cell lines the overall efficiency of gene transduction remains low @ 1-10%. This would be inadequate in the case of a gene that was directly toxic to cells notwithstanding the bystander effect. If the gene that was incorporated resulted in immune stimulation then it is possible that such a low rate of transduction would prove adequate.

In order to overcome this transduction inefficiency, combinations of replication-deficient and replication competent viruses have been evaluated(276).There remains the danger however with the use of the wild-type virus of creating a spreading systemic retroviral infection with its attendant dangers of inducing tumors through insertional mutagenesis. This fear has been realized in 3 monkeys which developed T-cell lymphomas of the thymus when retroviral vectors contaminated with replication competent viruses were used in transduction studies.

Adenoviruses

More recently adenoviruses have been used as viral vectors in transduction studies. Studies by four different groups(277-280) showed the feasibility of using replication-deficient adenoviruses to transfer genes in vivo. All used the E.coli LacZ gene as a reporter of successful transduction. Three of the groups used the Rous sarcoma virus long-terminal-repeat(RSV LTR) as a promoter while the remaining group used the early enhancer/promoter of the cytomegalovirus to drive expression of the LacZ gene.

The adenoviral vector has since been used to transfer other genes into various target cells including the α-1 antitrypsin gene into lung epithelial cells(281) and the thymidine kinase gene into malignant lung cancer cells(282).

The advantages of adenoviral vectors include the following :

(i) a broad host and cell-type range

(ii) growth to high titers

(iii) purification by density gradients

(iv) episomal location (little possibility of insertional mutagenesis)

(v) high level of expression of foreign genes

(vi) lack of demonstrable neurovirulence.

The disadvantages include the ability to accommodate only 6-8 kb of foreign genes as well as difficult gene manipulation. The long term safety of adenoviruses is unknown. Deletion of the early gene regions E1A/E1B results in a replication-defective virus but this may not completely inhibit viral replication which may pose a safety problem. A humoral immune response to the adenoviral vector has also been seen in animals in non-neural tissues which may interfere with transduction efficiency.

Viola, Ram et al (personal communication) have carried out both *in vitro* and *in vivo* studies using the adenovirus to transduce gliomas. They found high(~100%) rates of transduction of human glioma cell lines and also showed a prolonged period of gene expression(using the lacZ gene as a reporter gene)-as long as 3 months. They also carried out transduction studies of the adenoviral vector in a 9L gliosarcoma model in rats as well as the 9L meningeal carcinomatosis model also in Fischer rats. They found that almost all the cells expressed tumor at the growing edge of the tumor with X-gal staining detected in 50% of cells in the center of the tumor. A lesser transduction rate was seen with intrathecal administration of the adenoviral vector.

A recent study using the adenoviral vector carrying the HSV.tk gene in rat gliomas showed complete tumor resolution following subsequent treatment with gancyclovir. However ,these tumors were only $0.4mm^2$ on average and therefore the volume of tumor that needed to be transduced was relatively small(283).

One recent study compared the gene transfer efficiencies of a replication-deficient adenovirus, a replication-deficient retrovirus and a replication-conditional thymidine kinase-deficient HSV vector in 9L gliosarcomas both *in vitro* and *in vivo*(284).The lacZ reporter gene was used in these studies. They found that the retroviral and HSV vectors were selective for brain tumor cells while the adenoviral vector, whilst it transduced tumor cells, also transduced many normal brain cells. Gene transfer to multiple infiltrating tumor deposits with sparing of intervening normal brain was seen in one animal with use of the HSV vector. The HSV vector also produced extensive necrosis and selective inflammation in the tumor unlike the other two vectors.

Adenovirus Virology and Genetics

Adenoviruses(285-289) are simple DNA-containing naked capsid viruses that induce latent infection in the tonsils, adenoids, and other lymphoid tissues of humans. Most infections caused by adenoviruses in humans are acute and self-limited. Several serotypes of human adenoviruses cause undifferentiated tumors when inoculated into newborn hamsters, rats and mice.

Adenovirus virions are icosahedral structures 60 to 90nm in diameter (175×10^6 daltons) containing 252 capsomeres, which include 240 hexons which make up the faces, and 12 pentons which comprise the vertices. Each virion contains a single double-stranded DNA molecule, which varies in size from 30 to 38 kilobase pairs ($20\text{-}24 \times 10^6$ daltons), depending on the serotype. The linear DNA is associated with proteins which, together with 3 arginine-rich proteins, form the dense viral core which is visible as a unique structure on electron microscopy.

The host cell tropism of viruses is determined by specific cell receptors. The adenovirus is a non-enveloped DNA virus which is responsible for a significant number of human respiratory (7% of childhood respiratory infections) and gastro-intestinal (17% of all infant diarrheas) diseases.

The adenovirus has a 30 nm long fiber composed of 3 identical 62kD polypeptides which project from the each of the 12 vertices of the viral surface.

Following viral binding via its fiber protein and its corresponding cell surface receptor, adenovirus internalization occurs by receptor-mediated endocytosis. Following initial attachment of virus, clustering of receptors occurs. Attachment leads to interactions with the cell plasma membrane resulting in viral internalization. A host derived factor is necessary for viral entry as adenovirus binds to, but does not enter some cells.

The adenovirus 400kD penton base contains five Arg-Gly-Asp sequences. It forms a heterodimeric protein (penton) complex with the adenovirus fiber. A penton complex , with the N-terminal domain of the fiber inserted in its central cavity, is found at each of the 12 vertices. The distal C-terminal domain of the fiber terminates in a knob which projects from the virion and presumably contains the cell-binding region. Divalent cations are also required for virus internalization.

The penton base's repeating tripeptide sequence is also found in a number of cell adhesions (matrix) molecules such as fibronectin and vitronectin. The penton base RGD sequence binds to the α_v integrins (290)particularly $\alpha_v\beta_3$ and $\alpha_v\beta_5$ as do the above matrix proteins. Virus infection of cells can be inhibited by growing these cells on fibronectin and vitronectin surfaces which bind the α_v integrin receptors preventing binding of the adenoviral penton base. Therefore the susceptibility of a cell to adenovirus

infection may depend on both the presence of fiber specific receptors as well as the matrix to which the cell is attached. Soluble penton base causes adherent cultured cells to round up and detach. Cells with similar fiber and penton base receptors located in different tissue environments in vivo may exhibit significant differences in susceptibility to infection depending on the matrix protein(s) to which they are attached. This may be of great importance in their use as therapeutic vectors in brain tumors(see discussion).

Integrins are also implicated in the interaction of other viruses (picornaviruses, cocksackie and echovirus) with host cells as well as certain pathogenic bacteria(Yersinia and B.pertussis bind β_1 and β_3 integrins respectively).

Integrins play an important role in cell signaling processes including calcium mobilization, protein phosphorylation , cytoskeletal interactions and alterations in cytoplasmic pH. Thus viral binding to the integrin receptor could alter all these functions.

The adenovirus genome is highly conserved and therefore most of the 41 adenovirus serotypes use α_v integrins for infection even though some use different receptors for attachment.

The replication process of the adenovirus can be divided into well-defined early and late phases. During each phase different portions of the viral genome are transcribed into mRNA. The usual distinction between early and late synthesis is the onset of viral replication.

Following adsorption and penetration of the virus particle the final uncoating of the capsid occurs in the nucleus. The host cell's RNA polymerase II is used to transcribe the early viral genes into mRNA's. The full replication cycle takes 24-30 hours. The early mRNA's appear after 8 hours. Early transcription involves a complex set of genes that act as regulatory molecules controlling the expression of the late genes or that act as essential enzymes in the replication of viral DNA. The early mRNA is spliced, capped and polyadenylated after which it is transported into the cytoplasm. This mRNA is translated to form early gene products. The early proteins are transported back into the nucleus where they act as regulatory proteins during the early period. They help initiate viral DNA synthesis. The amount of viral genome required for early functions ranges from one-third to one-half the DNA.

E1A codes for three gene products which have multiple important uses including

(i) Activation and/or enhancement of transcription from other viral promoters.

(ii) Interacts with cell oncogenes such as *c-ras* to produce fully transformed phenotypes.

E1A functionally and structurally resembles the SV40-T, papilloma E2 and the herpes 1E genes.

E2A codes for a 22kD DNA binding phosphoprotein while E2B codes for a 72kD single stranded DNA binding protein that is required for the initiation of viral DNA synthesis.

The E3 region is non-essential in tissue culture but is conserved because of its function in modulating the host response to Ad infection. The 19kD product binds to MHC polypeptides and prevents their transport to and expression on the plasma membrane. This decreases T-cell recognition. A 14.7kD product inhibits the lysis of Ad infected cells by tumor necrosis factor. A third 10.4 kD protein binds to the EGF receptor.

E4 codes for a 11kD protein that binds to the nuclear matrix as well as a 34kD protein that forms a complex with the E1B 55kD protein in the nucleus.

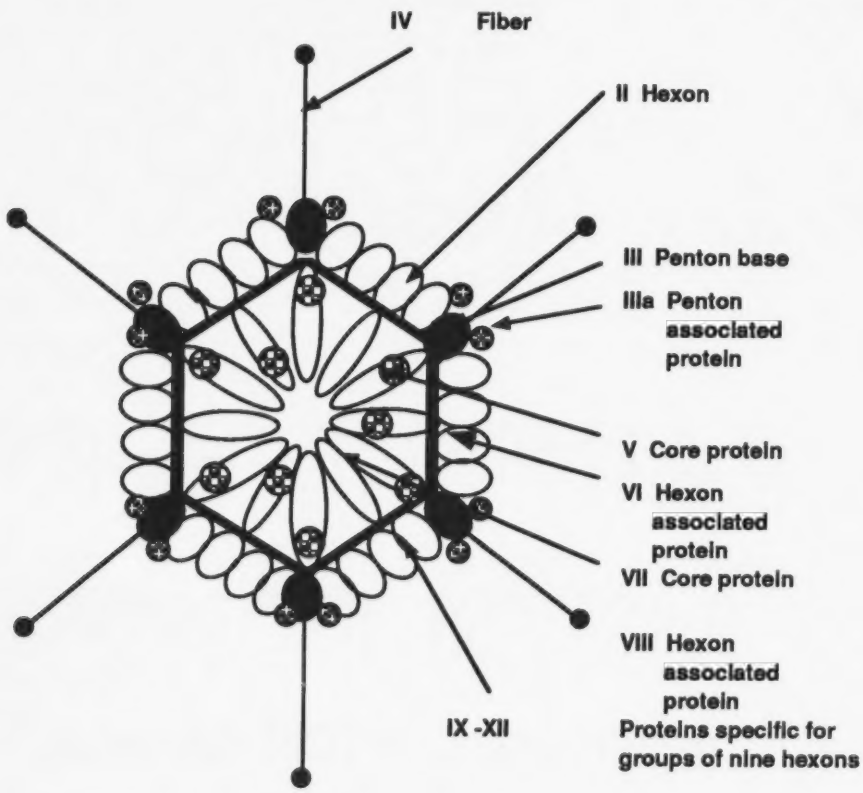
DNA synthesis proceeds once the early genes have been synthesized and the necessary enzymes for DNA synthesis are present. Adenovirus DNA sequences appear 5 hours post-infection(p.i.) in a large form integrated with host DNA. 8 hours later mature DNA molecules accumulate in the nucleus. A DNA polymerase replicates the linear double-stranded DNA. 2 cellular proteins are required for the initiation of replication; a 60kD type I topoisomerase and a 55kD protein that acts as a primer. In the non-lytic replication (transformation) cycle, only the early gene products appear and act to change the regulation of gene expression in the host cell.

At intermediate and late times, regions 2 and 3 are expressed. During late transcription , the portion of the viral DNA that was turned off during the early period is actively transcribed producing mostly the capsid proteins which are involved in the formation of new viral particles. These include protein II(hexon), III(penton base), IIIa(peripental hexon associated), IV(fiber), V(minor core), pVI(hexon associated, precursor), pVII(major core, precursor), pVIII(hexon-associated, precursor), IX(hexon associated). Capsid formation and the maturation of complete particles occurs in the nucleus after which they are released as naked viral particles without budding from the cell surface membrane.

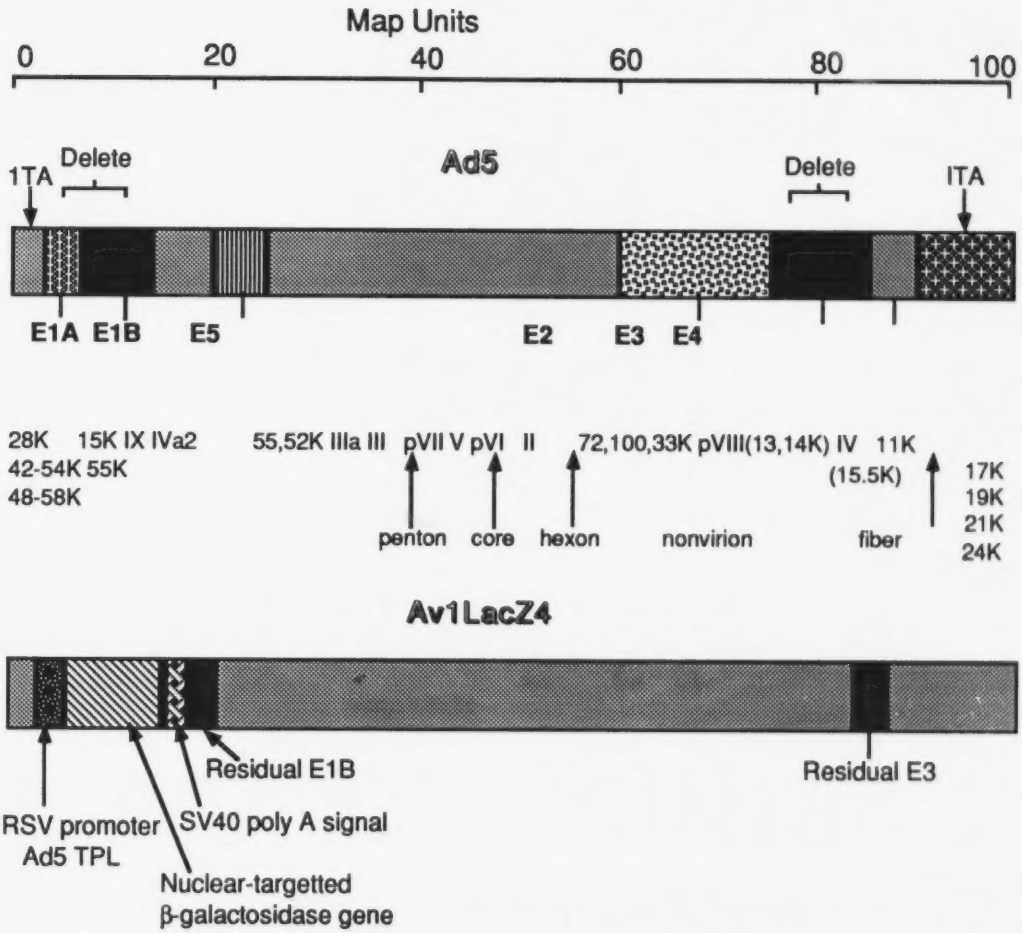
In the non-lytic cycle the cell is not destroyed and mature adenoviral particles are not produced. Instead ,in many adenoviruses (including Ad 5), the adenoviral DNA remains as an episome which is a circular DNA molecule which can replicate independently or with the cell's genome. The period of viral gene expression is therefore limited and the gene will not be transferred to the progeny during cell replication.

Some adenoviruses can remain latent in lymphoid tissue for as long as 24 months post-infection. Adenoviral DNA has been found in peripheral blood lymphocytes. These might be responsible for adenoviral infection in immunecompromised hosts as a result of

re-activation. An extreme example of latency is the stable integration of the adenovirus DNA with the host chromosome. Types 12,18 and 31 are highly oncogenic as a result of integration into the host gene with subsequent activation of adjacent cellular proto-oncogenes which culminates in the development of tumors(insertional mutagenesis).No adenoviral macromolecules(DNA, RNA, or proteins) have been found in studies of the etiology of various tumors. However, one group of investigators has found adenoviral nucleic acid in neurogenic tumors(291). This finding has not been replicated.



Schematic representation of the adenovirus particle
The Roman numerals refer to a polypeptide designation by Maizel et al.The corresponding common name for the proteins is also given.



The above two figures represent the genomic structure of the wild-type adenovirus serotype 5(top) and the genetically modified experimental adenovirus (bottom).

The 36,500 base-pair chromosome is divided into 100 map units. Viral proteins are designated by their weight in kilodaltons(K) or by Roman numerals(according to the classification by Maizel et al)(292, 293).

The Experimental Adenovirus

The experimental adenovirus used in the present study is the β-galactosidase Av1LacZ4 vector (Genetic Therapy Inc., Gaithersberg, MD). It is constructed from a genetically engineered serotype 5 virus. The E.coli nuclear targeted β-galactosidase gene is inserted into the E1A region in conjunction with a Rous sarcoma virus promoter and a Simian Virus 40(SV40) poly-A signal. The E3 region which is not essential for growth is deleted as is part of the E1A region. The vectors are grown in 293 cells which are human embryonic kidney cells which have been transformed into a continuous line by adenovirus type 5 (part of the viral genome has been incorporated into that of the host).The cells are infected at a multiplicity of infection(MOI) of 10 plaque forming units(pfu's)/cell. Growth of these replication-deficient vectors is possible because the constitutively active E1A region is available in these cells as a trans activating element. The viral particles so produced , are purified to yield final concentrations of 2-3 x 10^{11} pfu's/ml.

β-galactosidase

The E.coli β-galactosidase gene is used as a reporter gene of successful transduction. Its presence in transduced cells is detected using a suitable substrate, X-gal.

β-galactosidase enzyme activity is widely distributed in the cells and tissues of many mammalian species(294).The acid form of the enzyme is the most widely distributed form. It has a pH optimum of 3.5-5.5 and is responsible for the catabolism of glycolipids and mucopolysaccharides. The neutral form of the enzyme, primarily located in the intestine and kidney ,has an apparent pH optimum of 5.5-6.0. It plays a an important role in the hydrolysis of lactose in food.

The E.coli enzyme has a pH optimum of 7.3 whereas the endogenous mammalian brain enzyme has a pH optimum of 3.5-5.5. In the brain X-gal staining is observed in Purkinje cells of the cerebellum and to a lesser extent in many nuclear groups in the brain. However, even at the working pH used to detect the bacterial enzyme i.e. 7.5, background staining is seen in infiltrating macrophagic pericytes.

β-galactosidase (β-D-galactoside galactohydrolase) catalyses the following reaction.

β-galactosidases are inactivated in less than 1 minute @ 55°C. The enzyme is detected using X-gal substrate (5-bromo-4-chloro-indolyl β-D-galactoside)(295,

296).The enzyme liberates an indolyl compound from X-gal. Subsequent oxidation and self-coupling yields a blue indigo derivative which is insoluble and exceptionally stable. The substrate buffer is chosen to ensure an optimum pH for the bacterial enzyme and to provide suitable concentrations of NaCl and Mg^{2+} which are an activator and co-factor respectively of the enzyme.

Cells that have been transduced with this nuclear targeted β-galactosidase have dark blue staining nuclei. Some cytoplasmic staining will also be seen to a lesser extent.

The Bystander Effect

This is the term given to the phenomenon whereby virally transduced/modified cells pass on the toxic effect to adjacent non-transduced cells. This phenomenon has been best characterized in cells which have been transduced with the HSV.tk gene which results in sensitivity to the anti-viral agents gancyclovir and acyclovir with resultant cell death. This lethal effect is transmissible to surrounding cells. Thus tumor regression can still occur when only a fraction of the tumor mass has been transduced.

Several mechanisms have been postulated to help explain this phenomenon.

(i) Continued viral proliferation and infection in situ

(ii)Transfer of an integrated retroviral or adenoviral HSV-tk gene during mitosis

(iii)Transfer of the toxic purine analogue GCV triphosphate via gap junctions or apoptotic vesicles. Freeman et al(297) showed that the toxic effect was not due to a soluble factor as the effect was abolished when a population of HSV.tk +ve cells was separated from HSV.tk -ve cells by a filter membrane. They further went on to examine HSV.tk cells treated with gancyclovir and found them to undergo apoptosis which was characterized by cell shrinkage, cell detachment, vesicle formation and chromatin condensation. Using a lipophilic dye PKH 26 ,they showed that surrounding tumor cells took up the apoptotic vesicles generated by GCV mediated apoptotic cell death. The receptors involved in this uptake process may be cell type specific via surface structures such as the integrins which acts as the vitronectin receptor in neutrophils.

However a second school of thought believes that this transfer of a toxic product takes place via gap junctions(functional rather than anatomical gap junctions)(298).This has been shown via the transfer of a dye Lucifer yellow from transduced to non-transduced adjoining cells. *In vitro* studies using [3H]-labeled gancyclovir found that the radioactivity could be transferred from HSV.tk transduced cells to untransduced cells(299).Thus, only cells in direct cell-cell contact with the transduced cell will be affected by the bystander effect.

(iv)The hsv-tk enzyme itself may be transferred through these apoptotic vesicles or gap junctions(299).

(v) The transfer of hydrolases and/or other enzymes released by cells undergoing apoptosis.

(vi) Incorporation and expression of viral proteins may increase the antigenicity of tumor cells resulting in a heightened immune response. Hogquist et al (300)showed that processing and release of interleukin 1 occurs during apoptosis. Interleukin-1 is a potent stimulator of the immune system. This heightened immune response may be partially responsible for the bystander effect.

Given the biodistribution problem with the various vectors carrying the tk gene, the bystander effect will prove to be a useful phenomenon in the treatment of malignant gliomas.

CHAPTER 2. MATERIALS AND METHODS

Random source cats were quarantined for three weeks, conditioned and vaccinated against feline respiratory complex (feline rhinotracheitis, feline caliciviral disease) and feline panleukopenia as well as rabies. They were also given Pyrantel Pamoate for worms and Ivermectin for ear mites. They were fed ad lib and started on Cyclosporin A 20mg/kg/day orally in divided doses. The Cyclosporin A comes as an oral solution (Sandimmune, Sandoz Canada Inc.,) which is injected into commercially available clear gelatin capsules to formulate the correct dose. On some cats (n = 6) Cyclosporin A trough levels were obtained from venous samples on day 7.

After 10 or more days of Cyclosporin A treatment (this is the optimum period of pre-operative dosing based on previous studies in this laboratory)(302) the cats were fasted overnight and taken for tumor implantation.

Tumor Implantation

D-54 MG is a human glioblastoma derived cell line (courtesy of Dr.D.Bigner - Duke University) which is maintained in culture in RPMI 1640 with 10% fetal calf serum and antibiotics (Penicillin/Streptomycin/Amphotericin B). Passages 80 to 90 were used in these *in vitro* and i*n vivo* experiments.

The cats were given a pre-anesthetic cocktail of Buprenorphine 0.01 mg/kg atropine 0.04mg/kg and acepromazine 0.02mg/kg after which they were anaesthetized with 5% Isoflurane via a mask. The level of anesthesia was monitored by noting the respiratory rate as well as abolition of the blink reflex indicating an adequate level of surgical anesthesia. The cat was then intubated endotracheally and connected up to an anesthetic machine (Narkomed Anesthetic Systems, North American Drager Co.,Telford,PA) which provided both oxygen and Isoflurane. 500mg of IV Ampicillin was given pre-operatively as a prophylactic antibiotic.

The cat's head was shaved and placed in a stereotactic head-holder and secured firmly using ear and infra-orbital bars. The head is prepped with a Povidone-Iodine solution and suitably draped exposing the operative field.

A 2 cm midline incision was made centered over the approximate area of the coronal suture and the scalp retracted laterally. The periosteum was lifted off with a periosteal elevator to expose the coronal suture. Bilateral 3mm diameter burr holes were made using a dental drill , 5mm on either of the midline and 5mm posterior to the coronal

suture. The dura was coagulated with a bipolar cautery and a small incision made. A 10mm length of sterile polyethylene tubing (which has pre-cut side holes) was then passed over a 22G $3^1/_2$ G spinal needle and attached to a syringe on the stereotactic frame. It was then passed through the right burr hole, into the cortex to a depth of 8mm. The tube was left in place and the needle removed.

The D-54 MG cells which were growing in T125 culture flasks were removed by trypsinisation, spun down by gentle centrifugation (1000 rpm x 5 minutes) and resuspended in a 250μL volume of RPMI 1640 containing 1% methyl cellulose. A small aliquot(10μL) was drawn off and mixed with 90μL of trypan blue and the number of viable cells assessed in a counting chamber to ensure sufficient numbers for tumor implantation. 125μL of the tumor solution containing 5 - 10 million viable cells was injected into the lumen of the tube as well as into the cortex adjacent to the tube. A further 125μL was injected in two separate inoculations(next to each other) through the left burr hole. The cells were allowed to spill sub-pially. The holes were sealed with bone wax and the incision closed in two layers with 3-0 Vicryl and 3-0 Dermalon sutures. The cats were allowed to recover and closely monitored for the onset of neurological symptoms. Given the bifrontal location of these tumors, the neurological signs that were seen included circling, hemiparesis, refusal to feed, lethargy, pupillary dilatation, etc. They were kept on the same dose of Cyclosporin A post-operatively as well as amoxicillin 50mg orally twice daily for the first 5 days.

Imaging Studies

At 35 days post-implantation (or sooner if the cats showed any neurological deterioration) the cats were taken for an MRI scan of the head under Somnatol (barbiturate) anesthesia. The MRI scans included T_1 weighted (TR= 3 secs, TE= 26-104 msecs, 4 echoes of each slice) Gadolinium-DTPA enhanced and T_2 weighted (TR=500 msec, TE=26msec) images. If no tumor was seen on these images the animal was rescanned at 50 days post-implantation. If a tumor was seen on MRI scan, its exact location particularly in reference to the implanted tube as well as its dimensions was noted.

Virus Implantation

The cat was allowed to fully recover from this anesthetic, fed ad lib and approximately 2 days later was re-anesthetized using the afore-mentioned pre-anesthetic cocktail and Isoflurane. The head was shaved, prepped , draped and fixed firmly in the stereotactic frame. The previous midline incision was re-opened and the underlying burr

holes exposed. Subcutaneous tumor was often found spilling out from the burr holes. This was cauterized with bipolar cautery and removed to expose the margins of the burr hole. Using the dental drill with a longer cutting attachment, a craniectomy was carried out centered on the burr hole. The dimensions of the craniectomy were determined by the pre-operative MRI findings.

The adenoviral solution was prepared from stock solution (courtesy of Genetic Therapy Inc.,Gaithersberg,Maryland).The Av1LacZ4 BB7-4 containing 2.3 x 10^{11}pfu/ml and Av1LacZ4 BB 8-1 containing 7.5 x 10^{10}pfu/ml both carry the lacZ gene which codes for the E.coli β-galactosidase enzyme. The stock solution (kept frozen at -70°C) was thawed rapidly and diluted in PBS (10μL of virus in 100mL of PBS) to give a final working concentration of 2 x 10^{8}pfu per 10μL volume. This was then drawn into a Hamilton syringe and injected in 10μL aliquots into the area of the tumor. The injections were made 3 mm apart and at three separate depths at each site(2,5 and 8 mm). Any bleeding from the tumor was controlled with Gelfoam® which was also used to seal the craniectomy defects. The incision was closed in 2 layers with 3-0 Vicryl and 3-0 Dermalon. The cat was then housed in a special biocontained room and allowed to recover. It was closely monitored (twice daily) for any neurological deterioration.

The control group consisted of cats with tumors which were injected with a similar volume of PBS(~ 200μL).These cats grew tumor on both sides (hemispheres) and received the virus injection on one side and the PBS into the opposite hemisphere. The two groups were well matched in that each pair (virus vs. control) of injections occurred in the same animal with the same degree of immunesuppression, etc.

The effect on the transduction rate as a result of breaking down the tumor stroma prior to viral injection was assessed in two cats which underwent injection of proteases into the tumor prior to viral inoculation. In the first cat(G-7), 200μL of a sterile collagenase type IV solution was injected into the tumor bilaterally. The adenoviral solution was then injected immediately and 10 minutes after the collagenase on the left and right sides respectively.

F-127 was injected with 100μL of an enzyme cocktail consisting of Pronase and collagenase, into the anterior half of the tumor. An hour later the adenovirus solution was injected into both halves of the tumor.

G-23 underwent a tumor debulking using bipolar cautery and forceps to remove tumor tissue. The remaining tumor cavity was lined with Surgicel® which was then impregnated with the adenoviral solution. The surrounding margins were also injected with the virus solution. This was carried out in order to assess the effect, of decreasing the overall tumor cell burden prior to viral inoculation, on tumor transduction efficiency.

Brain Fixation And Sectioning

At 24 and 48 hours the cats were euthanized with an overdose of IV Euthanyl (a concentrated solution of barbiturate and alcohol). The chest was opened and an intra-cardiac catheter inserted into the left ventricle; a small opening was also made in the right atrium. Cold 4% formaldehyde was infused through the intra-cardiac catheter till the limbs stiffened indicating an adequate degree of fixation. The brain was removed and the area of the tumor exposed and blocked into 5-6 mm cubes which were rapidly frozen in Isopentane cooled in liquid nitrogen. The blocks were then cut into 10μM sections using a cryostat.

In two cats prior to euthanization an attempt was made to assess the efficacy of an intra-arterial mode of viral delivery. The cat was anesthetized and the neck shaved and prepped. The external carotid artery and its branches were dissected out as far distally as the angle of the mandible. The lingual, facial and thyroidal branches were tied off and a 24G IV cannula was introduced into the carotid artery distal to the take off of these branches. It was advanced as far distally as it would go and secured in place. 5cc of a 20% solution of mannitol was injected over a 5 minute period. This was followed 5 minutes later by an injection of trypan blue(5-10cc).The cat was subsequently euthanized and the brain removed.

Detection of Gene Expression

The frozen sections were incubated in the X-gal substrate which was prepared as outlined below.

Solution A was prepared by dissolving 10mg of IbGa(X-gal ; Gibco.PRL.Life Technologies Inc.,Gaithersberg,MD) in 0.5ml of dimethylformamide(20mg/ml).

Solution B was composed of 7ml of PBS containing 1.1M $MgCl_2$ to which was added 0.5ml of 50mM potassium ferrocyanide and 0.5ml of 50mM potassium ferricyanide.

Both solutions can be stored at -20°C for 3 months. The working X-gal solution was made by mixing 4.552 mls of Solution B with 100mL of Solution A.

The sections were incubated at 37°C for 24 hours. They were then washed in distilled water and counter stained with nuclear fast red for 5 minutes. The sections were dehydrated in an alcohol series, treated with Hemo-DE and mounted with Permount® and a coverslip.

The sections were examined under the high power of the microscope and the percentage of blue staining cells assessed in 10 high power fields. This gives the transduction rate of the virus.

In Vitro Gene Transfer

Cell cultures were derived from some of our own clinical patients. This was done as outlined below.

Specimens of grade IV astrocytomas fresh from the operating room were minced into pieces of about mm^3 and incubated with an enzyme cocktail consisting of collagenase (Gibco PRL,Life Technologies Inc,MD),DNAse (Sigma Chemical Co., St.Louis,MO) and Pronase(Boehringer-Mannheim,West Germany) for 30 minutes in a 37°C water bath. The tissue was filtered through a fine mesh ,dissolved in PBS and spun down (1000 rpm x 10 minutes).The cells were resuspended in several mls of SMEM(or Dulbecco's Modified Eagle's Medium) with 5% fetal bovine serum and a cell count carried out with trypan blue staining to assess the number of viable cells. The cells were cultured in DMEM/F12 by plating the cells at no less than 2×10^5 cells/$25cm^2$ flask.

6 different glioblastoma cell lines were tested for in vitro transduction(E-2, E-14, E-62, E-76, E-78 and E-80) as well as the D-54 MG cell line maintained in RPMI 1640. The D-54 MG cell line was the only cell line used for tumor implantation and therefore in vivo studies. A comparison of the in vitro transduction rate of the adenoviral vector in D-54 MG cells with the transduction in other human glioblastoma cell lines was necessary in order to generalize any results obtained with the D-54 MG tumors in vivo to all human glioblastomas i.e. to ensure that the D-54 MG cells were not significantly different phenotypically in comparison to other glioblastoma cell lines in terms of sensitivity to adenoviral transduction.

The 7 different cell lines were plated onto 16 well culture plates. Once a monolayer of cells was obtained, the medium was changed to one containing 1% fetal bovine serum in order to inhibit further cell growth/division. A cell count was carried out on one of the chambers using trypan blue. The adenoviral stock solution was thawed and diluted in the growth medium to yield a concentration of approximately 2×10^8pfu/ml of medium. 250µL of this adenoviral solution was added to each chamber to give a virus to cell ratio of approximately 1000 pfu's : 1 tumor cell. In each 16 well plate 2 chambers were inoculated with medium alone containing 1% fetal bovine serum but no virus solution. A further two chambers had half the viral concentration inoculated. The cells were incubated with the virus at 37°C for 1 and 4 days. The medium was removed and the wells rinsed in PBS. A fixative solution containing 2% formaldehyde/ 0.2%

glutaraldehyde was then added to each well and left on for 10 minutes at 4°C. The wells were again rinsed in PBS following which, 200μL of X-gal solution was added to each chamber. This was left at 37°C for 4 hours(this has been found to be the optimum period of incubation in our lab).The X-gal substrate was removed and the cells fixed with cold 70% ethanol for one hour. The chamber walls were broken off and the slides counter stained with nuclear fast-red, dehydrated in an alcohol series and mounted in Permount® (Fischer Scientific Ltd.,) and cover-slipped. The slides were then examined under the microscope and the percentage of blue staining cells in 10 high power fields assessed. This gave an indication of the transduction efficiency of the adenoviral vector for each of the cell lines *in vitro*.

Chapter 3. Results

In Vitro Studies

The adenoviral transduction efficiency was assessed in 7 glioblastoma cell lines and was found to be 90-100% in all the cell lines tested. There were different intensities of blue staining of the cells. The different cell lines also showed a variation in their morphological appearance both before and after viral inoculation(Fig.11 and 12). The D-54 MG cell line has been cultured for a long time and is currently undergoing its 95+ passage in culture. It tends to be less differentiated i.e. fewer processes and also less stellate in appearance. This may be a result of prolonged culture environmental influences.

Two other cell lines, E-2 and E-62 which tend to grow in culture at rates comparable to that of D-54 MG also showed a more bipolar morphology. The remaining 4 cell lines tended to be more stellate in appearance though to varying degrees. These cell lines also grow more slowly under standard cell culture conditions.

The D-54MG, E-14, E-80 and E-62 cells showed both light and dark blue cell nuclei. There was a spectrum of blue intensity among the cells within each cell line. The ratio of light blue to dark blue cells varied among the cell lines with the E-14 having the largest proportion of light blue to dark blue cells(2:1) and the E-62 showing the smallest number of lighter staining cells(1: 20).

Using different MOI's (multiplicities of infection), it was found that at a ratio of 1000 pfu's/ cell the transduction rate was almost 100%. When the MOI was reduced to 100 pfu's/cell the transduction rate fell to 60-70%(Fig 13). With a MOI of 10 pfu's/cell the transduction rate was less than 5%.

The same transduction rate was seen at both 1 and 4 days post viral inoculation. The cell lines were not followed long enough to assess both long term viability of transduced cells or the period of gene expression.

In vivo studies

Twelve cats harboring intracerebral tumors had intratumoral inoculations of the adenoβ-gal vector. Since many cats had bilateral tumors, these contralateral tumors were used as controls and were injected with PBS solution alone. A further 2 tumors were injected with defective virus (virus had been frozen and re-thawed more than twice); in vitro studies using these same viruses failed to show any β-gal expression. These tumors failed to show β-gal expression and hence were included as controls.

Transduction rates of 80-100% were seen in the immediate vicinity of the viral injection(Figs.14-18). This decreased to about 50-60% further out and fell to essentially

0% approximately 500-600μM circumferentially around the site of injection(Fig.15).This was true in all 12 tumors. In some cases where satellite tumor nodules were found removed from the main tumor mass, transduction of these tumor cells was also seen with sparing of intervening normal brain tissue. There was negligible transduction of normal neurons and glia in areas of brain surrounding tumor(Fig.21).However, if the viral injection was directed into the brain, transduction of normal astrocytes, ependymal cells, endothelial cells as well as choroid plexus cells was seen(Fig.22).It thus appears that , when virus is injected into tumor near the interface between tumor and brain, the tumor cells appeared to take up the virus preferentially acting as a viral sponge. Another interesting feature noted on these sections was the presence of large numbers of small pre-pyknotic looking cells that were not transduced with the adenovirus. These mostly occurred near the sites of viral injection.

Thirteen of the cats were euthanized at 48 hours, seven at 24 hours and one cat at 4 days post viral implantation. There was no difference in the transduction rate at 1,2 and 4 days. Three cats that underwent viral-implantation immediately after the MRI imaging, remained lethargic and drowsy the day after viral injection. This may have been due to two consecutive anesthetics (both pentobarbital and Isoflurane) having been given within a short period of time. Examination of the brains showed no evidence of hemorrhage or gross swelling to account for these symptoms. One cat however did undergo delayed (24 hours) viral implantation and became lethargic and developed a cranial nerve III palsy and hemiparesis. Autopsy showed the presence of a small hemorrhagic clot around the site of injection as well as hydrocephalus resulting in critically elevated intra-cranial pressures. The one cat that received the enzyme cocktail injection into half the tumor, was found to be lethargic, drowsy and not eating or drinking. Autopsy showed a moderate sized (3 mm diameter) blood clot at the site of injection. This effect was most likely attributable to the matrix and tissue destroying properties of the enzyme cocktail.

Of the twelve cats injected with the adenoviral vector, only two developed symptoms directly attributable to the viral injection. The remaining cats tolerated the viral injection very well without any deterioration in their neurological status. Histological examinations of the brains showed no evidence of hemorrhage, edema or gross demyelination (although the Weil's method used to stain myelin is too imprecise to show small defects in demyelination along the site of injection). Electron microscopy may perhaps help to pick up these more subtle changes. There was evidence of mechanical disruption of the tumor as a result of the viral injection with a moderately heavy small cell infiltration(most likely lymphocytes) around the margins of the needle tract.

A significant area of tumor between the viral injection sites was found to be not transduced with the virus i.e., there was a finite limit to viral penetration into the surrounding extra-cellular matrix(Fig.16).

In areas of tumor growing along the leptomeningeal surfaces of the brain and down the sulci, a much more impressive transduction of tumor cells was seen. Large areas of this surface tumor showed 80-100% transduction with the adenoviral vector (Figs.19 & 20).Unlike the intracerebral tumors, a larger area of the tumor was found to be transduced indicating a difference in susceptibility to viral transduction between the two locations.

In one cat, a tumor debulking was carried out with subsequent injection of the recombinant virus into the margins of the remnant tumor ; the residual cavity was also lined with Surgicel® soaked in the adenovirus solution. On histological examination, however, very little tumor was left following the debulking procedure allowing areas of normal brain surrounding the cortex to be transduced .

In the two cats that underwent a protease injection into the tumor prior to the viral injection , the adenovirus solution was found to be inactivated and therefore no transduction was seen. A small hemorrhagic clot was seen at the site of injection of the enzyme cocktail.

Tumor Implantation Results

The mean dose of Cyclosporin A was 21.65mg/kg/day in two divided doses. The Cyclosporin A trough levels drawn on 9 cats on day 7 post treatment is shown in Table 5. A comparison of the dose per kg of Cyclosporin A administered versus the resulting trough level showed there to be a moderate degree of correlation (Spearman correlation co-efficient = 0.76) which was significant at the 5% but not the 1% confidence level. This is in keeping with the fact that drug levels are not only dependent on the weight of the cat but also on other pharmacokinetic variables.

Thirty-two cats were implanted with tumor (18 male: 14 female). The D-54 MG cell line was used for tumor implantation in all except 2 cats where the E-62 cell line was used. Thirty cats developed tumors. Two animals were euthanized early as a result of pneumonias and thus did not have sufficient time to grow a tumor. All cats that developed a tumor had at least a leptomeningeal surface component with or without a intracerebral component.

Fifteen cats did not have a tube implanted at the time of tumor implantation while the remaining 17 cats did have a right sided tube implanted. Twenty-six cats underwent an MRI scan prior to viral implantation or euthanasia whereas the remaining six cats died or

were euthanized due overwhelming symptoms which precluded their going for an MRI imaging procedure.

The mean cross-sectional area of the tumors of the cats implanted with the tube was 135.9mm^2(S.E of the mean = 21.3mm^2). In cats without a tube implant, the mean cross-sectional area of the tumor was 43.58mm^2(S.E of the mean = 9.32mm^2)(Tables 1 & 3). Using the Wicoxon rank sum test for non-parametric data, the difference in cross-sectional area was found to be statistically significant($p = 0.001$). Cats with a tube had tumors which were on average 3.12 times the cross-sectional area of those without a tube implant. The individual data for each cat is shown in Tables 2 and 4. The distribution of tumor cross-sectional areas in the 2 groups is shown in Fig.I.

Eleven cats developed neurological symptoms. The commonest presentation was with a hemiparesis resulting in an ataxic gait. Less frequent presentations were a monoparesis, lethargy, anorexia and in one case a 3rd nerve palsy.

The time to tumor presentation either on MRI scan or as a result of the development of neurological symptoms was compared in the 2 groups. Cats with a tube implant presented earlier (mean = 26.94 days ; S.E.M.= 1.424) than cats without a tube (mean = 54.545 days;S.E.M. = 8.967 days). Using the Wilcoxon rank sum test, this difference was found to be statistically significant($p = 0.003$).This is shown in Fig.II. There was a subgroup of cats which developed sizable tumors within 21 days of implantation. These were special pathogen free cats(SPF) which had been reared in isolation and hence were immunologically naive compared to the normal population.

Six cats developed symptoms directly attributable to Cyclosporin A treatment. Two cats developed a pneumonia(one bacterial(Pasturella) and one presumed viral as no pathogen was isolated) while a further two developed disseminated toxoplasmosis affecting most of the major organs but surprisingly sparing the brain. Two cats developed gingival hyperplasia necessitating a switch in their diet to softer food.

There were three anesthetic deaths. Two of these deaths occurred during MRI scanning under Saffan anesthesia. This particular anesthetic agent was found to be difficult to titrate and hence a successful switch to pentobarbital was made in subsequent studies.

On gross and histological examination, three cats were found to have hydrocephalus as well as a tumor. Hydrocephalus was probably caused by tumor cells having been partially injected into the ventricles or to leptomeningeal seeding of tumor.

A comparison of the size of tumor seen on MRI scan with the Cyclosporin A trough level in the 9 cats in whom levels were drawn shows there to be a poor correlation(Spearman correlation co-efficient = 0.05).However a certain minimum

immunesuppression is needed in order for the tumor to seed and grow as demonstrated in previous studies in this laboratory(302).

In the first part of the experiment when the animal model was being refined, the animals were kept on Cyclosporin for varying periods of time post-operatively following which they were weaned off the Cyclosporin A. Seven cats were weaned off the Cyclosporin after periods of 15,20 and 25 days post-implantation. However only two(28.6%) of these cats grew tumors of an appreciable size as seen on MRI imaging. These two cats were without Cyclosporin A for 16 and 46 days. The rest of the cats required resumption of Cyclosporin A treatment with subsequent tumor re-implantation. These cats did show tumors on histological examination but these were almost exclusively small surface nodules without any appreciable cortical component.

The remaining cats were kept on Cyclosporin A for a mean of 26.3 days.

Histological examination

Histological examination of these tumors showed growth primarily along leptomeningeal surfaces and within sulci i.e. within easy access of CSF with subsequent invasion of the cortex(Figs 5-7).Tongues of tumor tissue could be seen invading the surrounding cortex mostly clustered around small blood vessels. Remote satellite nodules of tumor were also seen again mostly clustered around small vessels(Fig 8).

The tumors consisted of masses of polymorphic cells with large irregular nuclei often showing mitotic figures. There were also areas of necrosis as well as widespread neovascularization with endothelial proliferation, scattered giant cells and multi-nucleated cells(Fig 9). However, tumors from cats later on in the series i.e. those which had been kept on continuous Cyclosporin post-operatively had more fusiform cells that were often arranged in fascicles i.e. more like a gliosarcoma than a glioblastoma. These features are in keeping with the adult pilocytic variant of the glioblastoma multiforme. If it is a gliosarcoma it is possible that the sarcomatous cells are derived from malignant transformation of fibroblasts. Immunohistochemical studies of these tumors, however, have shown them to be mostly GFAP negative with some areas that are vimentin positive. However these same cells in culture prove to be GFAP positive.

One interesting feature seen on the sections from the cats that were weaned off the Cyclosporin A was the presence of large numbers of small dark round cells infiltrating the tumor(Fig.10). These cells, in all probability lymphocytes or microglia, were seen scattered throughout the tumor especially in relation to the numerous small blood vessels dispersed through the stroma of the tumor. Thus a strong rejection phenomenon was

demonstrated. Specific characterization of these cells i.e.(CD^{4+} CD^{8+} Natural killer cells) is yet to be performed.

Chapter 4. Discussion

Tumor implantation studies

The initial part of the study was devoted to refining the existing animal glioma model. The major disadvantage of the human glioma model stems from the fact that the animal is chronically immune-suppressed and hence any therapeutic trials would occur against this background of chronic immunesuppression. In addition, Cyclosporin A has other properties apart from immunesuppression, which would make its concurrent use in tumor therapeutics research undesirable.

Cyclosporin A inhibits the growth of many cells in culture including the growth of tumor cells. Thus although it allows the tumor to seed and grow by suppressing the host's immune responses, it also retards growth of these tumors in the long term. Cyclosporin A binds to a specific receptor called cyclophilin which is an isomerase (or rotamase).This complex then binds to, and inhibits the Ca2+ and calmodulin dependent protein phosphatase, calcineurin A(303,304). In the absence of Cyclosporin A binding, stimulation of the cell (e.g. the T-cell, by occupancy of the T-cell receptor), results in a kinase cascade which eventually results in a rise in intracellular Ca^{2+} levels. This in turns activates calcineurin which leads to the translocation into the nucleus of the cytoplasmic subunit of the T-cell restricted transcription factor, NF-AT. Inhibition of calcineurin by the cyclosporin-cyclophilin complex causes inhibition of NF-AT as well as OAP, AP-3 and NF-κB(to a lesser extent).This results in a decrease in the DNA-binding affinity and transcriptional activating properties of these transcription factors. This has been best characterized for the IL-2 gene in T-cells. Cyclosporin A however, also blocks the transcriptional activation of other cytokine genes, such as interleukin 3(IL-3) and IL-5. It also blocks the production of tumor necrosis factor α by B cells. IL-3 is an autocrine oncogenic regulator in some tumor cell lines(e.g. the v-H-*ras* oncogene transformed mast cells). Cyclosporin A has been shown to inhibit growth of such cell lines by destabilizing the IL-3 mRNA(305). IL-2 is also a growth stimulatory cytokine. Another study has shown that Cyclosporin A can inhibit cell growth via a transforming growth factor β (TGF-β)- mediated mechanism. Cyclosporin A increases the production of TGF-β which in turn inhibits cell growth(306). Thus prolonged Cyclosporin A treatment could result in inhibition of further tumor cell growth.

Anecdotally, one cat which was taken off the Cyclosporin A after 30 days of treatment as it was thought not to be growing a tumor, was found to harbor a massive tumor when it was euthanized at 80 days. This would suggest that once a critical size of

tumor is reached, in a relatively immunologically privileged site as the brain, further immune suppression was unnecessary. Perhaps the shedding of soluble tumor antigens which act as decoys, binding the effector cells and molecules at a distance from the tumor may have been responsible.

As outlined in the Introduction section, in order for a proper cellular immune response to be mounted against a foreign antigen, the antigen has to be presented not only in conjunction with a MHC class II molecule but also in the presence of a co-activator, the B-7 molecule present on professional antigen presenting cells. In the absence of the B-7 antigen, the Th cells go into an anergic state accepting the foreign antigen as self. However many gliomas do not express MHC class I and/or II molecules nor do they have the B-7 molecule. Thus the immune response is dampened. It's possible that once a large enough number of tumor cells are present, many of the circulating Th cells will be rendered anergic leaving only a small proportion to mount an ineffectual immune response.

Cyclosporin A is also a inhibitor of the P-glycoprotein pump and like verapamil will enhance the therapeutic effect of some chemotherapeutic agents (212,307,308). The P-glycoprotein pump expels many chemotherapeutic agents from the cell reducing their effective cellular concentration and is one of the causes of multiple-drug resistance. Thus concurrent treatment with Cyclosporin A would produce results which would have to be excluded in order to separate the true effect of a particular drug under trial.

However, attempts to refine the model any further were only partly successful. The doses of Cyclosporin A used in these studies were less than those used in the original model(21.65mg/kg/day vs 24 - 30 mg/kg/day). Two cats were successfully weaned off the immunesuppression after a period of 20 days post-implantation. The remaining cats had to be re-started on Cyclosporin A and/or re-implanted with tumor. Histological examination did show signs of rejection with a heavy small cell infiltrate most likely representing tumor infiltrating lymphocytes. Thus the following conclusions can be drawn from these studies.

(1) A minimum period of Cyclosporin A treatment is required post-implantation for adequate tumor growth to occur. This period appears to be at least 25 days post-implantation. There was the sub-group of SPF cats which grew tumors relatively quickly. Whether this represents a picture of facilitated xenograft acceptance is unclear. It is possible that with less antigenic exposure the immune system remains relatively undeveloped both anatomically and functionally such that subsequent challenge with a foreign antigen results in acceptance or lack of rejection of that antigen. T-cell reactivity

tests would have to be carried out on the T lymphocytes derived from these SPF cats and compared with those from a normal cat.

(2) A period of Cyclosporin A treatment is required pre-operatively in order to facilitate tumor implantation and seeding. Studies have shown that within 72 hours of treatment with Cyclosporin A there is adequate T-cell suppression to prevent the mounting of an effective rejection process. However in the previous study(302), a minimum of 10 days of pre-operative dosing was required to facilitate adequate tumor growth.

(3) A smaller dose can be used effectively perhaps reducing the attendant side-effects of prolonged Cyclosporin A treatment. Above a minimum dose(approximately 20mg/kg/day) further increments in dosage does not appear to cause an increase in the size of tumor seen. If this is further correlated with Cyclosporin A trough levels, a trough level of 1000-2000 ng/ml can be taken as indicative of adequate immunesuppression .

(4) The Cyclosporin A trough levels at any given dosage are mostly dependent on the weight of the cat. However, other pharmacokinetic factors may be important and peculiar to each animal. The trough level is therefore the best indicator of adequate dosing. As well, a T-cell reactivity assay has been developed (i.e. looks at the end-effect of Cyclosporin A action) and would be the best possible measure of immunesuppression.

(5) The brain is not as immune privileged a site as once thought. This previous belief has been increasingly challenged in recent years and is further supported by this study in which cats weaned off Cyclosporin A showed a heavy lymphocytic infiltration indicating an active immune process in the CNS. This response may be secondary to the blood-brain barrier breakdown which is commonly seen in high grade gliomas and which would facilitate the recognition and binding of tumor antigens by circulating peripheral lymphocytes. It is possible that the various cells that effect the immune response are generated within the CNS itself; glial cells can act as antigen presenting cells while the microglia(which may originate in the peripheral lymphoid tissue) may then differentiate into the various effector cells. The presence of the B-7 antigen on the glial cells and the CD4, and CD 28 molecules on the microglia should be looked for in order to resolve this issue. The CD8 molecule has been found to be expressed in the microglia infiltrating brain tumors(116).

A further refinement to the original model was the use of a implanted polyethylene tube to facilitate tumor growth. This model change resulted from observations made on the previous model where it was noted that the initial tumor seeding and growth occurred in the subarachnoid spaces either on the cortical surface or within sulci. This finding suggested a role for CSF in facilitating tumor growth by perhaps

providing nutrients until adequate neovascularization made the tumor self-sufficient. The polyethylene tube(with side holes) acted as an artificial sulcus filled with CSF in which tumor cells would seed and grow out through the side holes and invade the surrounding cortex.

A second problem tube insertion addressed that of tumor localization. A significant discrepancy between the location of tumor on MRI and its actual location on histological section was invariably found. This geometric shift in the position of various structures within the cortex has been previously documented in clinical studies. The magnitude of the shift appears to depend on many factors including the field strength of the magnet, the TE and TR times, the use of paramagnetic contrast agents and the size and composition(water content) of the object being imaged. This geometric shift would cause localization problems when attempts were made to inject the viral vector intratumorally using MRI imaging derived co-ordinates.

Cats with tube implants grew tumors which were on average at least 3 x larger than in animals without tubes. In all cases the tumor grew around the implanted tube with subsequent invasion of the surrounding brain. The silastic tubes were well tolerated.

Using the position of the tube as a reference point, the position and extent of the tumor could be more easily determined. This facilitated the second phase of the study which involved the injection of a viral transduction vector.

In vivo transduction studies

The transduction studies using the various glioblastoma cell lines showed the experimental adenoviral vector to be an efficient agent for the transfer of specific genes to gliomas, at least in vitro. Nearly a 100% transduction rate was seen in all the cell lines tested. There were differences in the intensity of blue staining noted both between cell lines and within the same cell lines. Some cells showed an unmistakable dark blue nuclear staining while others showed a lighter blue impregnation of the nucleus.

The proportion of dark blue staining cells to lighter staining cells varied from cell line to cell line. The was also a variation in cell morphology between the cell lines both before and after viral inoculation. The D-54 MG cells particularly tended to take on a more rounded morphology. Whether these various changes and cell line peculiarities represents a true difference in sensitivity to adenoviral transduction is uncertain. Certain factors may help explain this differential sensitivity :

(1) This phenomenon may represent either a difference in the number of viral gene copies inserted into the cell or else may represent differences in transcriptional and translational activity between the different cells. Studies using bromo-deoxyuridine

labeling of cells(43) in the S-phase has shown the different cell lines to have different Proliferation Indices(from 18-30%).The PI also varies within a cell line depending on the plating density and the population density of cells at the time of BrdU labeling. Those cells which were actively dividing showed more active protein synthetic capabilities with more transcriptional and translational activity than those in the G_0 phase of the cell cycle. These cells may thus produce larger quantities of the β-galactosidase enzyme from the inserted viral genes than their more quiescent neighbors. As well, the occasional cell that was not transduced may have been resistant to adenoviral infection.

(2) The adenovirus binds to specific cell surface receptors via its fiber molecule and is subsequently internalized by interacting with the α_v integrins($\alpha_v\beta_1$ and $\alpha_v\beta_3$) on the surface of cells. In order for a cell to be susceptible to adenoviral transduction it must express both the specific cell surface receptor for the fiber molecule as well as the particular α_v integrin. Thus, different glioma cell lines may have differing quantities or even absence of one and/or both molecules to account for the differences seen.

The α_v integrins also act as receptors for various matrix proteins particularly vitronectin and fibronectin but not collagen. The presence of these proteins in high concentrations competitively inhibits binding of the adenoviral penton base complex to the α_v integrin receptor resulting in resistance to adenoviral transduction. Conversely the presence of large numbers of adenoviral particles could interfere the binding of cells to the various matrix proteins resulting in the cells rounding up and coming off the culture plates. In summary, the different glioblastoma cell lines may therefore ;

(i) Express different proportions of the specific cell surface receptor and αv integrins

(ii) not possess one and/or both these molecules or,

(iii) produce varying quantities and proportions of the various extra-cellular matrix proteins especially fibronectin and vitronectin.

In vivo transduction

The *in vivo* transduction studies demonstrated that the adenovirus is an effective transduction vector for the transfer of specified genes into glioblastoma cells. Injections into the interface between tumor and normal brain resulted in the tumor cells being exclusively transduced with sparing of the normal cortical neurons and glia. The tumor thus appears to act like a sink or a sponge, drawing the adenoviral particles. This differential sensitivity may again be explained by the factors postulated above. The normal cortical neurons and glia are post-mitotically quiescent with very low proliferation indices and thus they may fail to get transduced or if transduced may fail to express the

gene product. The cortical neurons and glia may express smaller quantities of the adenovirus binding receptor and the α_v integrins in comparison to the adjacent tumor cells. It is postulated that the stroma of the normal cortex is made up of different extra-cellular matrix proteins (especially more collagen) in comparison to the tumor stroma again leading to a preferential transduction of the tumor.

When injections of the virus were made into normal cortex ,neurons and glia were transduced and expressed the β-galactosidase enzyme. The same was true of endothelial cells, ependymal cells and cells constituting the choroid plexus. Thus the adenovirus does not exclusively transduce tumor cells, although a differential gradient has been demonstrated between the tumor cells and the normal neuropil. This gradient may be very important in future clinical studies.

Preferential tumor cell transduction demonstrated in the present study is an important factor in our ongoing and future therapeutic studies. Adenoviral mediated transfer of the therapeutic herpes virus-thymidine kinase gene renders these cells sensitive to the anti viral agents acyclovir and gancyclovir. Gancyclovir is converted exclusively(see Introduction) by viral thymidine kinase to the monophosphate which is then sequentially phosphorylated by the cellular thymidine kinase to the triphosphate which is the active form of the drug. This GTP molecule acts as an inhibitor of DNA polymerase II and causes chain termination by substituting as a purine analogue;the two processes result in early cell death. Only transduced cells would be sensitive to gancyclovir. However as a further caveat, only mitotically active cells express large quantities of cellular thymidine kinase (which is an essential enzyme for DNA synthesis and therefore cell replication).Thus normal neurons and glia being post-mitotically silent have negligible quantities of the enzyme i.e. they are unable to convert the gancyclovir monophosphate to the active triphosphate form and would therefore not be sensitive to gancyclovir induced cell death. Shen et al (283) showed that injection of an adenoviral vector carrying the hsv-tk gene followed by gancyclovir treatment did not result in any cortical damage or neurotoxicity.

Thus the adenoviral vector carrying the hsv-tk gene would seem ideally suited for treating glioblastomas.

The transduction rate in areas of tumor growing along leptomeningeal surfaces and within sulci seems to be much higher approaching 100%; this transduction rate is also maintained over a larger surface area. Reasons for these results include :

(1) Easier access of the adenoviral particles to the tumor cells from the CSF

(2) A higher proliferation index of the leptomeningeal tumor compared to the cortical tumor because of lesser constraints to growth and replication of the cells (to be studies using BrdU labeling indices).

(3) Differences in expression of α_v integrins and the fiber molecule receptor.

(4) Smaller quantities of fibronectin and vitronectin especially as a proportion of the overall stromal protein content. This would result in less competition for the adenoviral binding sites with a resultant greater transduction. Again this hypothesis would have to be tested by immune-staining the stroma of both cortical and leptomeningeal tumor with antibodies to these various stromal components.

(5) Physical factors such as intratumoral pressure may help explain the difference in transduction rates, with the leptomeningeal tumor having lower interstitial pressure gradients compared to intracortical tumor.

This vector carrying the hsv-tk gene when given intra-thecally would be ideally suited for treating leptomeningeal deposits of tumor such as in patients with medulloblastoma with leptomeningeal spread. This form of treatment would be most welcome in the pediatric setting where irradiation of the developing brain results in significant neurological damage. One patient with medulloblastoma has obtained a 3 year disease free survival following intra-thecal treatment with a retro-viral vector carrying the hsv-tk gene.

The extent of transduction following an injection of virus is limited to about 500-600μM circumferentially around the site of injection. In order to cover a moderate sized tumor such as seen in every day clinical practice would require a multitude of injections spaced mm apart in a 3-dimensional array. Thus bio-distribution of the virus is a major drawback with the current use of adenoviral vectors. Previous studies using the retroviral vector carrying the hsv-tk utilized rat gliomas in a rat model. The viral vector injection was carried out a few days after the tumor inoculation using stereotactic methods. The tumor cell burden in these cases was small as was the extent of the tumor that needed to be transduced(271, 276, 309, 310). A more recent study using the adenotk vector in a murine glioma model reported cures of gliomas injected with the virus with subsequent gancyclovir treatment(283).However, the mean cross-sectional area of these tumors was only 0.4mm2. Thus these early promising results should be examined in the cold light of reality i.e. they should be tempered by the fact that the size of tumors tested is many degrees of magnitude smaller than the garden variety glioblastoma. Another factor to be borne in mind is the fact that many of these studies used the C6 rat glioma and the 9L gliosarcoma both of which have high proliferation rates compared to human glioblastomas.

If indeed the Proliferation Index impacts on the transduction rate, then the results in these rat models may not be easily or accurately transposed to the *in vivo* clinical situation.

Our model, though far from ideal, still provides a truer testing ground for animal therapeutic trials. The results from this study of adenoviral transduction therefore, more closely approximate the results that might be obtained when tested on human patients harboring glioblastomas.

Various other forms of viral delivery and/or adjunctive treatments should be assessed in order to achieve a greater percentage of tumor cell transduction. These might include the following;

(1) Intra-arterial viral delivery in conjunction with pharmacological blood-brain barrier modification. This particular mode of drug delivery has been utilized in order to achieve high concentrations of the chemotherapeutic agent within the tumor with minimal amounts reaching the systemic circulation(see Introduction). Basically the internal carotid artery is catheterized and an infusion of 25% mannitol given in order to reversibly open up the endothelial tight-junctions(this takes place to a greater extent in the tumor blood vessels than in normal brain since the barrier is already dysfunctional in the former). Infusion of an adenoviral solution after blood-brain barrier modification would follow. A further refinement is the use of intra-carotid histamine at a concentration of 10μg/kg/min(311). Histamine has been shown to be more selective in opening up the blood-brain barrier within areas of tumor with relative sparing of the surrounding normal brain. In order to further minimize extra-tumoral distribution of the virus the catheter could be advanced peripherally such that the infusion could selectively perfuse the tumor. Future studies will address this approach. The internal carotid circulation in the cat does not lend itself well for selective catherization as the internal carotid artery is mostly a fibrous cord in the adult cat. Most of the internal carotid circulation including the circle of Willis is fed by branches of the external carotid artery, especially the internal maxillary and ascending pharyngeal artery. Attempts were made in this study to selectively catheterize the external carotid artery beyond the take off of the lingual, superior thyroid and facial arteries with subsequent trypan blue injections following a mannitol infusion. Blue staining of the ipsilateral cortex was seen ; there was also blue staining of the ears as the internal maxillary also supplies this area before forming a rete internum from which the circle of Willis is constituted. Catheterization of the most distal branches would entail skull base surgery. An alternate approach would be to use a monkey tumor model which possesses a similar cerebral arterial anatomy to human. It is also possible that the adenovirus will transduce the endothelial cells of the blood vessels rather than enter the tumor. If the adeno-tk vector were used in this situation with subsequent gancyclovir

treatment, this could result in endothelial cell death, vessel thrombosis and possible tumor infarction.

(2) Delivery of the adenovirus solution by convection bulk-flow techniques. Various studies have shown that the tumor interstitial pressure can be quite high compared to the surrounding cortex such that it imposes a physical barrier to the movement of substances into the tumor(312).This increased interstitial pressure may be due to the presence of leaky blood vessels in combination with an absence of a fluid drainage system which results in the local accumulation of fluid and other substances. It has been shown that large areas of the cortex may be perfused with various solutions using convection bulk-flow without any adverse effects on the brain(313,314). This is to be assessed in the current model using a Harvard infusion pump to deliver an adenoviral solution into the area of the tumor over a several hours. Whether or not significant improvement in the biodistribution of the vector will occur , with improved transduction rates, is yet to be determined.

(3) Apart from the increased tumoral interstitial pressure, the stroma itself imposes a barrier to the movement of various substances including viral particles. Thus destruction of the extra-cellular matrix prior to viral infusion has a certain intuitive appeal. The stroma in glioblastomas contains many different matrix proteins including collagen, fibronectin, vitronectin, laminin, etc. Various metalloproteinases may be used to break down this stroma. Collagenase IV acts on type IV collagen which is found almost exclusively in the basement membrane of blood vessels(315).Other collagenases act on other substrates as do Pronase and DNAse. This was tried in two cats where the tumor was first injected with the protease and subsequently injected with virus. Unfortunately, defective virus stock was used making any results uninterpretable. This idea is to be actively pursued in the future. In order to ensure that these enzymes themselves don't damage the viral particles, *in vitro* testing of viral activity following exposure to these various enzyme cocktails is currently being addressed.

The transduction of tumor cells appears to be inhibited by the presence of large quantities of fibronectin and vitronectin in the stroma. Thus it would make intuitive sense to reduce the quantities of these stromal proteins to facilitate tumor cell transduction. Thus the use of these various proteases may prove to be doubly useful.

(4) As discussed earlier, the transduction rate and especially the degree of gene expression may be related to the particular phase of the cell cycle in which any individual cell is at any given time. Various agents may be used to increase the percentage of cells in the G_2-M phase or conversely reduce the number of cells in the G_0 phase. Estramustine is an estradiol based anti-microtubule drug which binds to tubulin and/or microtubule

associated proteins causing depolymerization of microtubules with resultant disruption of the mitotic spindle(249, 316).This results in mitotic arrest with accumulation of cells at the G_2-M phase. This produces a synchronized population of cells which are more sensitive to irradiation and perhaps also viral transduction. Estramustine may thus be given prior to viral injection to try and improve transduction efficiency. Taxol also causes mitotic arrest with sensitization of cells to irradiation(317-319). More recently taxol has been incorporated into biodegradable polymers for use interstitially in brain tumors(320).

(5) The *in vivo* studies showed the presence of non-transduced pre-pyknotic cells in various areas of the injected tumor. These pre-pyknotic cells though metabolically quiescent may still be re-activated when nutritional conditions improve; these cells may then replicate producing a recurrent tumor. Various agents may be used to improve oxygenation within hypoxic areas of the tumor.

Several methods have been utilized in an attempt to improve tumor oxygenation including:

(i) Hyperbaric oxygen

(ii) Perfluorochemical O_2-carrying emulsions such as Fluosol in conjunction with a high O_2 atmosphere(321).

(iii) Pharmacological manipulations to increase blood flow and/or oxygen off-loading.

(iv) Hemoglobin(Hb) solutions-studies have shown that the IV administration of an ultra-pure polymerized bovine Hb solution with normal air breathing decreases the percentage of severe hypoxia seen in 9L gliosarcomas from 49% to 24% and also increases oxygenation throughout the tumor(322).

This adjuvant treatment may result in improved viral transduction as was seen when 4 different alkylating agents were used with resultant increased survival in animals bearing intra-cranial 9L gliosarcomas(322).

There are various hypoxic markers which may be used to delineate hypoxic areas of tumor . [^{3}H]-misonidazole adducts appear to localize to well oxygenated regions of the tumor(323).The reactive species, an intermediate stage in the enzymatic reduction of the nitro group to the amine, requires oxygen to reverse the first reductive step and hence is dependent on oxygen. Misonidazole could thus act as a marker for delineating hypoxic areas of the tumor. A comparison with the viral transduction rate would then either confirm or refute the theory that hypoxic cells are more resistant to transduction.

(6) Tumor debulking may still play an important role in the management of glioblastomas by reducing the volume of tumor left to be transduced. In the one cat in which this was carried out prior to viral inoculation, very little residual tumor was seen

with transduction of many areas of adjoining normal cortex. However, this might not pose much of a problem given the selective action of gancyclovir on rapidly dividing tumor cells with sparing of post-mitotic neurons and glia.

(7) The adenoviral solution may perhaps be allowed to diffuse into the tumor over a longer period of time by placing it in an Ommaya reservoir in contact with the tumor may result in a larger volume of transduction.

(8) A multi-needle injector/syringe apparatus has been developed in conjunction with the Biomedical Design Department of the University of Alberta.It contains 18 25G needles which are placed at different depths and approximately 2mm apart over a 1cm diameter circular area. The configuration allows for the viral solution to be injected through all ports simultaneously resulting in a more uniform transduction of the tumor.

An alternate approach to this bio-distribution problem would be the use of replication competent adenoviruses which are genetically attenuated to be non-virulent. These vectors may be used alone or in combination with replication competent adenoviruses in order to cause a spreading infection within the tumor. Even if the infection was to spread into the adjacent normal brain, the transduction of normal brain would not be a problem if the tk gene were used in conjunction with gancyclovir, for the reasons previously outlined.

Producer cells producing large quantities of this adenovirus over a sustained period of time could perhaps be used akin to their use with retroviruses. Thus far genetically altered fibroblasts have been used as producer cells. The tumor cells themselves have been tried as producer cells with the hope that, as they replicate and invade, they would carry the virus with them thus helping to transduce a larger volume of tumor. This has not been borne out in practice as the tumor cells were phenotypically altered by the genetic manipulations such that they lost their invasive capabilities. An alternate type of producer cells may be the macrophage or lymphocyte which are inherently capable of migrating through tissues. These cells also tend to tolerate gene insertions and manipulations well. Adenoviruses often go into latency in lymphoid tissue and adenoviral DNA has been found in peripheral blood lymphocytes especially B-cells.

Possible adverse sequelae

(i) **Replication competence**-The adenoviral vector used in these experiments is assumed to be replication-deficient. However, some residual replication-competence may exist especially where the missing E1A region's function may be provided by a host cell. Some cells especially lymphoid tissue may carry latent adenoviral particles including the adenoviral DNA which may then provide the missing E1A function. Its also possible that

genetic recombination could theoretically occur in the latter cells resulting in the production of replication-competent viruses.

(ii) **Meningoencephalitis**-While adenoviruses mostly cause respiratory and gastrointestinal infections, they may on occasion cause meningoencephalitis especially in the immunecompromised host. Several adenoviruses have been found in the CSF including types 3, 5, 6, 7, 7A, and 12. Adenovirus encephalitis in the normal host usually occurs in association with severe systemic infection and has been associated with epidemic adenovirus type 7 pneumonia in children(324-326). In the immunesuppressed host adenovirus sepsis can occur causing lethal pneumonia, hepatitis and renal insufficiency. There have been numerous case reports of adenoviral encephalitis in the setting of immunesuppression. A patient with malignant lymphoma had grossly evident degeneration and hemorrhage in the inferomedial temporal and occipital lobes(327). Microscopically, large basophilic intra-nuclear inclusions were seen in neurons which on electron microscopy proved to be adenoviral particles. There was also sub-pial mineralization, marked gliosis, petechial hemorrhages, perivascular lymphocytic infiltrates and necrosis. Adenovirus type 32 was isolated from a brain biopsy in this patient. In another patient who had undergone bone marrow transplantation for acute myelogenous leukemia, similar histological changes were seen not only in the temporal lobes but also in the amygdaloid nuclei, hippocampus, hypothalamus and brain stem(328).

In patients with acquired immune-deficiency syndrome(AIDS), meningo-encephalitis secondary to adenoviral infection has been reported. The adenovirus seems to have an affinity for the ependymal lining as well as the sub-ependymal region resulting in an ependymitis with sloughing of the ependymal lining of the ventricles. This affinity for ependymal cells was confirmed in this study, where transduction of ependymal cells was seen following viral inoculation into the ventricular system. Periventricular gliosis has also been noted. In one pediatric patient, adenovirus type 5 was identified by in situ hybridization in the ependymal and sub-ependymal regions(329).Intracerebral inoculation of human(unattenuated) adenovirus type 7 produces significant ependymitis. Another patient with AIDS was noted to have symmetric bilateral degeneration of the corticospinal tracts, frontopontine fibers and cerebellar white matter(330).This patient had an adenovirus positive CSF culture. Although no choroid plexus involvement was seen in these patients, the current study found evidence of transduction of the choroid plexus in the cats. Thus the adenovirus may also cause a choroid plexitis similar to that seen with cytomegalovirus infection.

Thus meningoencephalitis remains a real risk both in this cat model due to the induced immunesuppression, as well as in human patients with their attendant immunesuppression especially given their depressed T-cell function.

(iii) **Induction of tumors**-The adenoviral DNA stays as an episome and therefore is not at risk of causing insertional mutagenesis. However the affinity of the adenovirus for the ependymal and sub-ependymal regions may be disturbing, given the results of one study (291)where simian adenovirus type 7 has been shown to induce tumors of paraneural origin in newborn hamsters, including microscopic tumors just below the ependymal lining.

(iv) **Post-viral phenomena**- There is a theoretical risk of causing an allergic post-viral encephalomyelitis through immune mechanisms. These cats are immunesuppressed and therefore may not show these immune sequelae.

Concluding remarks

Thus although the adenoviral vector is an efficient transducing agent for the transfer of therapeutic genes into glioma cells both *in vitro* and *in vivo*, it has some limitations. The most important of these is the finite geometric limit of viral transduction following a viral injection into tumor. To what extent the bystander effect will compensate for this short-coming remains to be studied using the adenotk vector. If the bystander effect is mediated through gap junctions, then only cells in anatomical contact with transduced cells will be affected. However if it is partly due to an augmented immune response to the presence of virally transformed cell antigens then a larger area may be encompassed by the bystander effect. The adenoviral vector used in these studies lacks the E3 region which normally helps down-regulate the host's immune response to adenoviral infection. Its absence would result in a stronger immune response.

In summary, viral mediate gene therapy is therefore not the last word in the treatment of glioblastomas. At present, many hurdles remain to be overcome. Perhaps gene therapy should be usefully combined with other treatment modalities in order to try and improve patient survival.

Gene therapy is very much in its infancy; delivery of a specific gene to all tumor cells including those remote from the parent tumor mass remains, as yet, an unattained goal in the treatment of malignant astrocytomas.

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CATS WITHOUT IMPLANTED TUBES

CAT NO.	TUMOUR CROSS-SECTIONAL AREA (mm^2)	TIME TO PRESENTATION (days)
F-99	28	46
F-49	48	95
F-33	50	69
F-96	54	28
F-53	27	99
E-68	125	27
E-66	20	25
F-34	10	60
F-37	15	91
F-38	26	25
F-39	80	35
F-47	40	29

Table 1. This table shows the cross-sectional area and the time to presentation (either on MRI scan or through neurological symptoms)for cats not implanted with a tube.

CAT NO.	PRESENTING SYMPTOMS
F-99	ataxic,dragging **L** leg
F-49	none
F-33	none
F-96	ataxic,dragging **R** leg
F-53	anorexic,gingival hyperplasia
E-68	ataxia,lethargy,dragging **L** side,pyrexia
E-66	lethargy,anorexia,**L** 3rd nerve palsy
F-34	ataxia,cold,anorexic
F-37	episodic circling,falling to the **R**
F-38	**L** hemiparesis,lethargy
F-39	none
F-47	none

Table 2. The above table details the presenting symptoms,if any, for the group of cats without implanted tubes

CATS WITH IMPLANTED TUBES

CAT NO.	TUMOUR CROSS-SECTIONAL AREA (mm^2)	TIME TO PRESENTATION (days)
F-92	104	34
F-93	150	37
G-5	180	23
G-7	375	22
F-91	90	32
G-6	65	24
F-115	190	33
G-4	104	28
F-120	90	26
F-119	50	22
F-127	100	28
F-116	150	19
G-18	130	none*
G-19	125	21

Table 3. The above table shows the cross-sectional area and the time to presentation (either through the onset of neurological symptoms or on MRI scan) of cats that were implanted with a polyethylene tube prior to tumour cell innoculation.(* this cat died from a respiratory tract infection prior to the MRI scan)

CAT NO.	PRESENTING SYMPTOMS
F-92	ataxic,coughing
F-93	cough,anorexia(prob. a resp.tract infection)
G-5	none
G-7	none
F-91	cough,mild fever
G-6	none
F-115	ataxia,**R** leg weakness
G-4	none
F-120	gingival hyperplasia
F-119	pyrexia of unknown origin
F-127	none
F-116	none
G-18	**R** pupil dilated,lethargic,unable to stand
G-19	died from anaesthetic

Table 4. The above table details the constellation of symptoms shown by cats with implanted polyethylene tubes

CAT NO.	CYCLOSPORIN A TROUGH LEVEL (ng/ml)
F-99	4522
E-68	2896
E-66	6034
F-34	1518
F-37	2207
F-38	1355
F-39	1699
F-97	1374
F-47	2003

Table 5. Cyclosporin A trough levels on selected cats.

Fig.I.

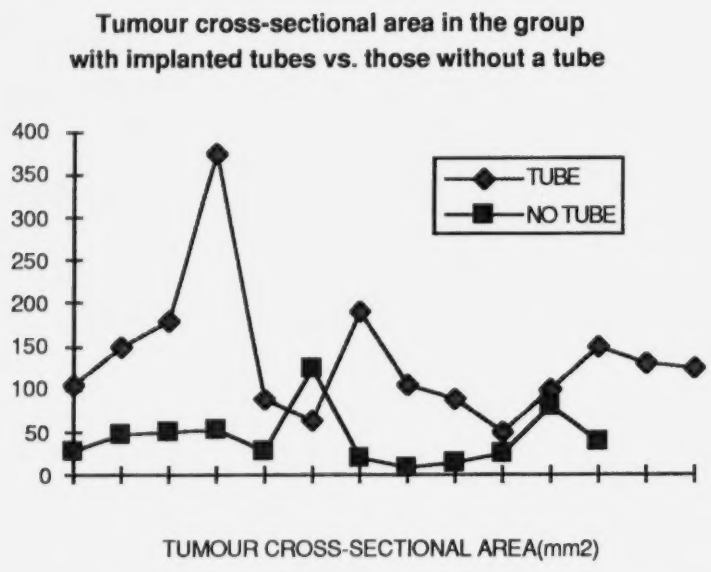
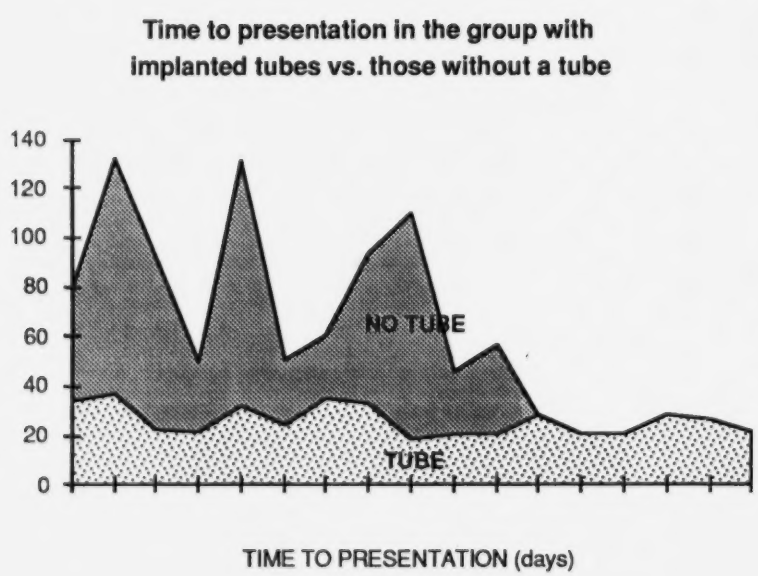

Tumour cross-sectional area in the group
with implanted tubes vs. those without a tube
400
350
300
250
200
150
100
50
0
TUBE
NO TUBE
TUMOUR CROSS-SECTIONAL AREA(mm2)

Fig.II.


Time to presentation in the group with
implanted tubes vs. those without a tube
140
120
100
80
60
40
20
0
NO TUBE
TUBE
TIME TO PRESENTATION (days)

LEGEND TO ACCOMPANYING ILLUSTRATIONS

Fig.1. A Gadolinium-enhanced MRI scan (T_1 weighted image) of cat G-6 showing bilateral tumours. The right sided tumour (open arrow ⇨) is surface-based, growing from the leptomeningeal surface and down a sulcus; the left sided tumour (arrow ➡) is deeply seated. Both tumours enhance uniformly with the paramagnetic contrast used (Gadolinium-DTPA) indicating some degree of breakdown of the blood-brain barrier.

Fig.2. This is a computer-aided colour enhanced version of Fig. 1. The tumour is indicated by the arrows ➡ .

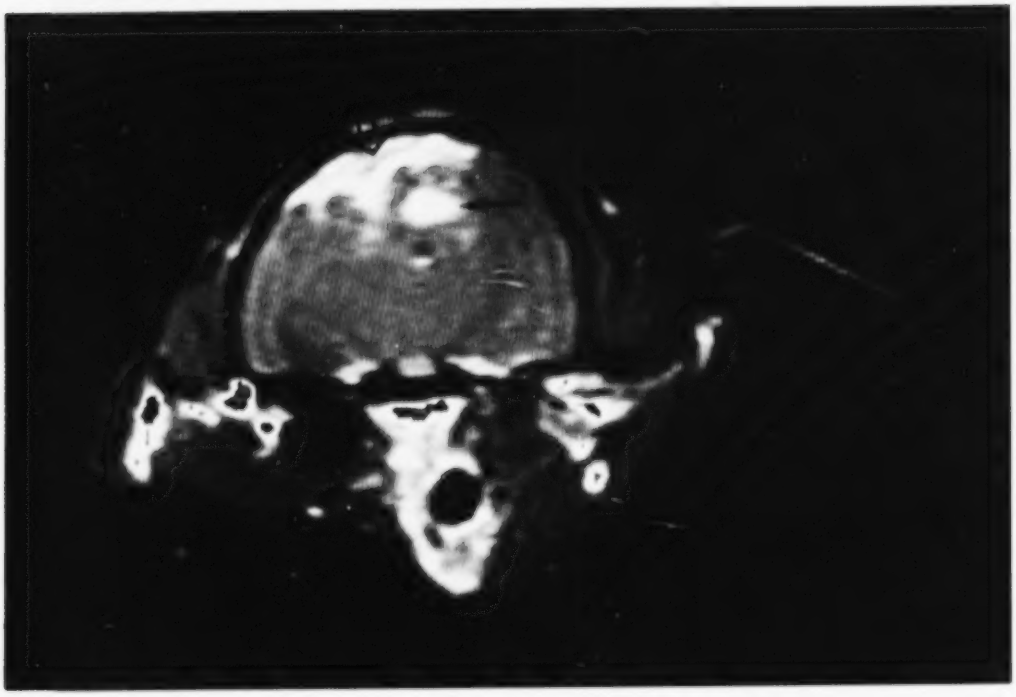


Fig.1. Gadolinium enhanced MRI scan(T_1 weighted) of cat G-6

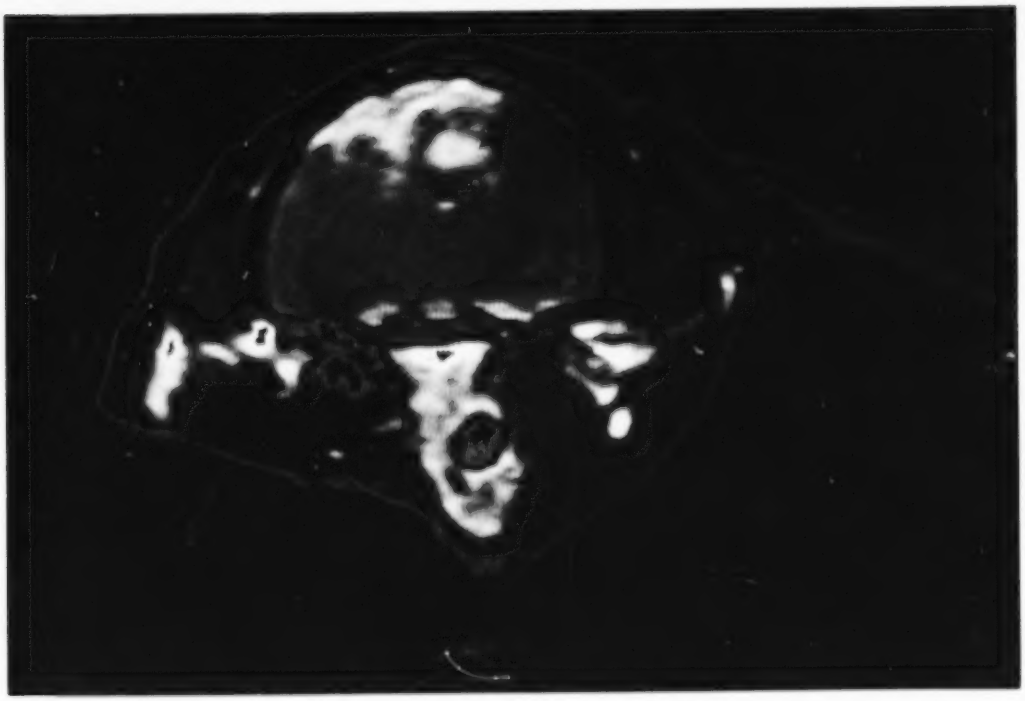


Fig.2. A computer-aided colour enhanced version of Fig.1

Fig.3. A Gadolinium-enhanced MRI scan (T_1 weighted image) of cat G-7 showing bilateral surface based tumours(arrows ➔).There is some enhancement of the overlying sub-cutaneous tissue indicating tumour infiltration into this area.

Fig.4. A Gadolinium enhanced MRI scan (T_1 weighted image) of cat F-90 showing a right sided tumour that is surface based and is growing around the implanted tube. Cats with implanted tubes grew larger tumours on average than cats without tubes.

Fig.3. Gadolinium enhanced MRI scan(T_1 weighted) of cat G-7

Fig.4. Gadolinium enhanced MRI scan(T_1 weighted) of cat F-90

Fig.5. Hematoxylin & eosin stained paraffin section of cat E-68 showing bilateral tumours.There is both leptomeningeal surface tumour(long arrows ⟶) as well tumour growing down a sulcus(short arrow ⟶).This cat developed ataxia and was euthanised.

Fig.6. Hematoxylin & eosin stained paraffin section of cat F-39 showing bilateral deep tumours arising from the inter-hemispheric fissure(arrow ⟶).This cat had been succesfully weaned off Cyclosporin A after 20 days post tumour implanation. This cat had been completely asymptomatic neurologically but died during the scan as a result of the anesthetic.

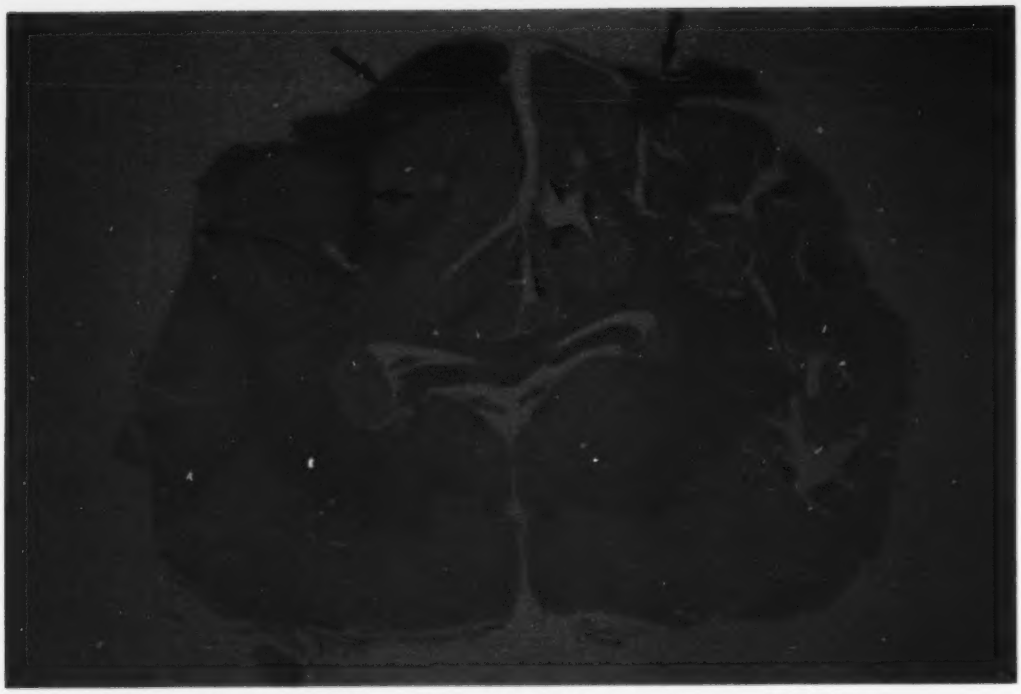


Fig.5. H & E stained paraffin section of E-68 showing tumour (x 10).

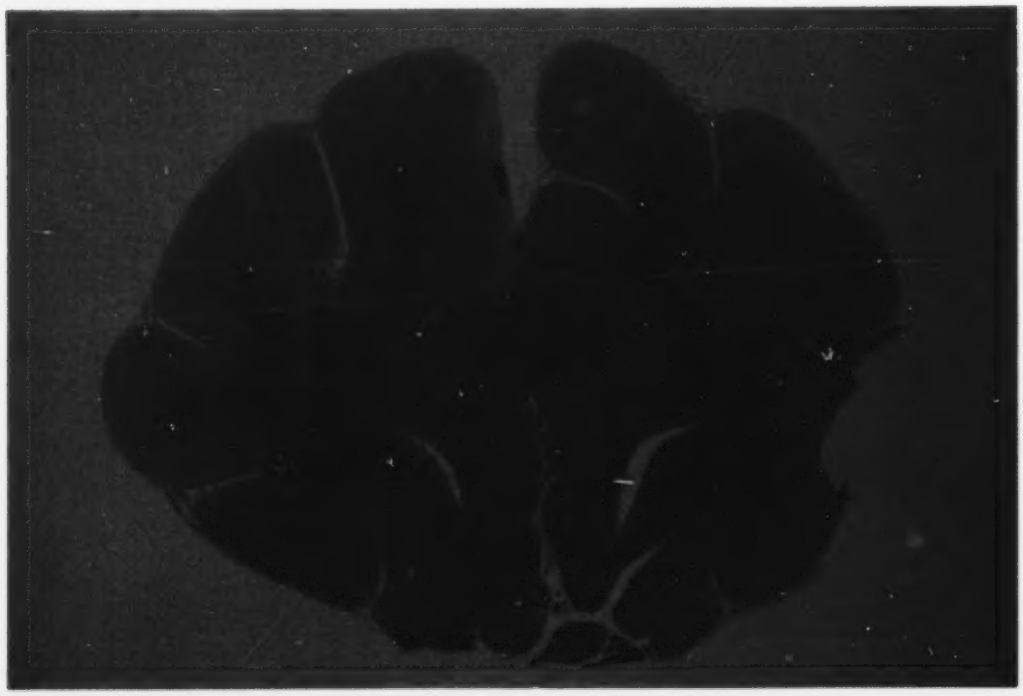


Fig.6. H & E stained paraffin section of F-39 showing tumour (x 10).

Fig.7. A higher power view of Fig.6. showing the 'kissing tumours'.The darker areas within the tumour represent areas of lymphocytic infiltration.This cat had been successfully weaned off its immmunesuppression and therefore the former may represent an attempt at immune rejection of the xenografted tumour.

Fig.8. A hematoxylin & eosin stained section showing an area of infiltrating tumour. Small islands of tumour(arrows ➔) may be seen infiltrating into the surrounding cortex.Most of these malignant cells are grouped around small blood vessels.

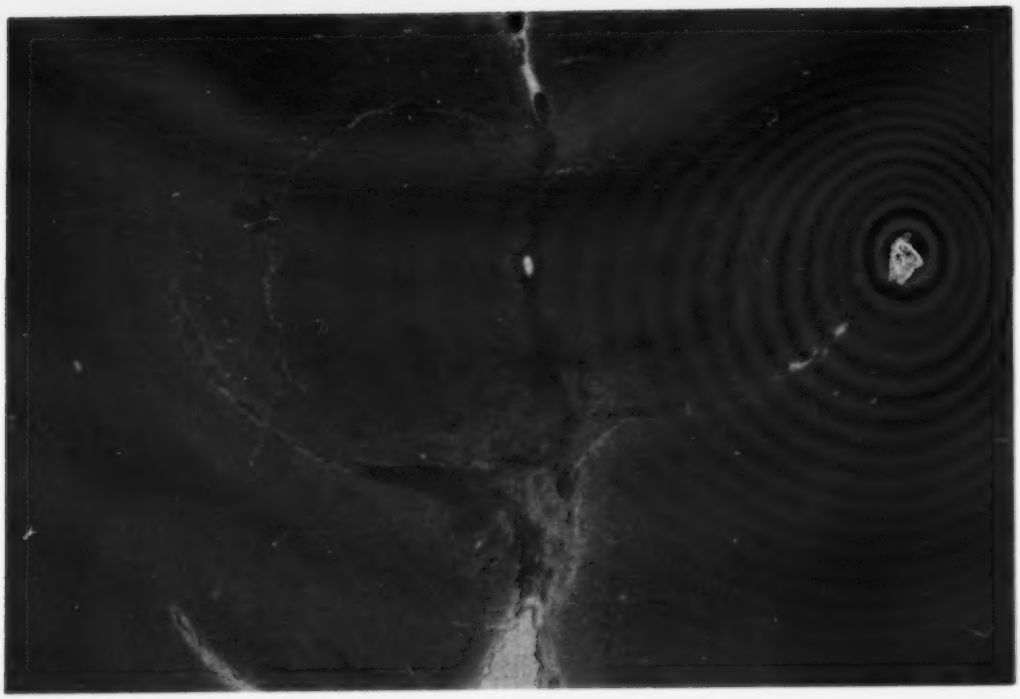


Fig.7. Higher power view of Fig.6. (x 40)

Fig.8. H & E stained section showing infiltrating tumour (x 100).

Fig.9. A high power view of an H & E stained paraffin section from cat F-53 showing masses of malignant cells with pleomorphic nuclei.Some mitotic figures are discernible.This tumour has many of the features of a glioblastoma multiforme.

Fig.10. A high power H & E section from cat F-39 which had been weaned off its Cyclosporin A after 20 days post-tumour implantation.Large numbers of small,dark round cells can be seen infiltrating throughout the tumour. (arrows ⟶).This may represent an attempt by the animal's immune system to reject this xenografted tumour.It stresses the fact that the CNS is not as immune-privileged a site as was once assumed.

Fig.9. H & E section of a glioblastoma multiforme from F-53 (x 400).

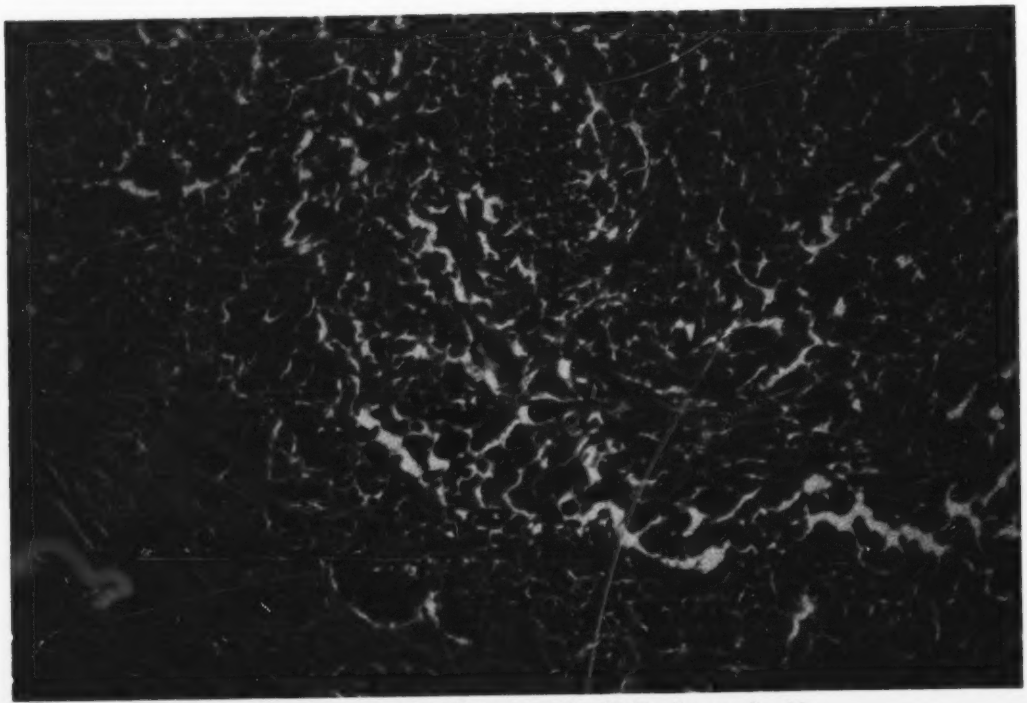


Fig.10. H & E section from cat F-39 (weaned off Cyclosporin A).

Fig.11. This is an X-gal stained section showing transduction of D-54 MG glioma cells *in vitro* .The reporter gene used is the nuclear localising β-galactosidase gene whose presence in transduced cells is indicated by the dark blue nuclear staining (arrows ➔).

Fig.12. An X-gal stained section showing transduced E-76 cells *in vitro*.The cells have the characteristic appearance of astrocytes.

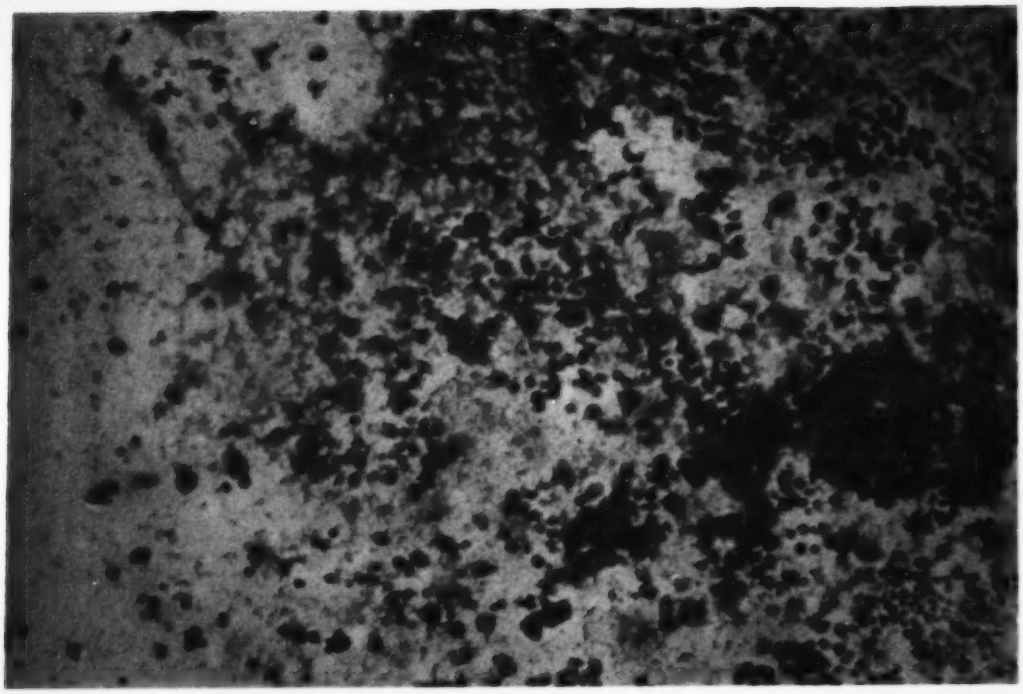


Fig.11. Transduced D-54 MG cells *in vitro* (X-gal stain).

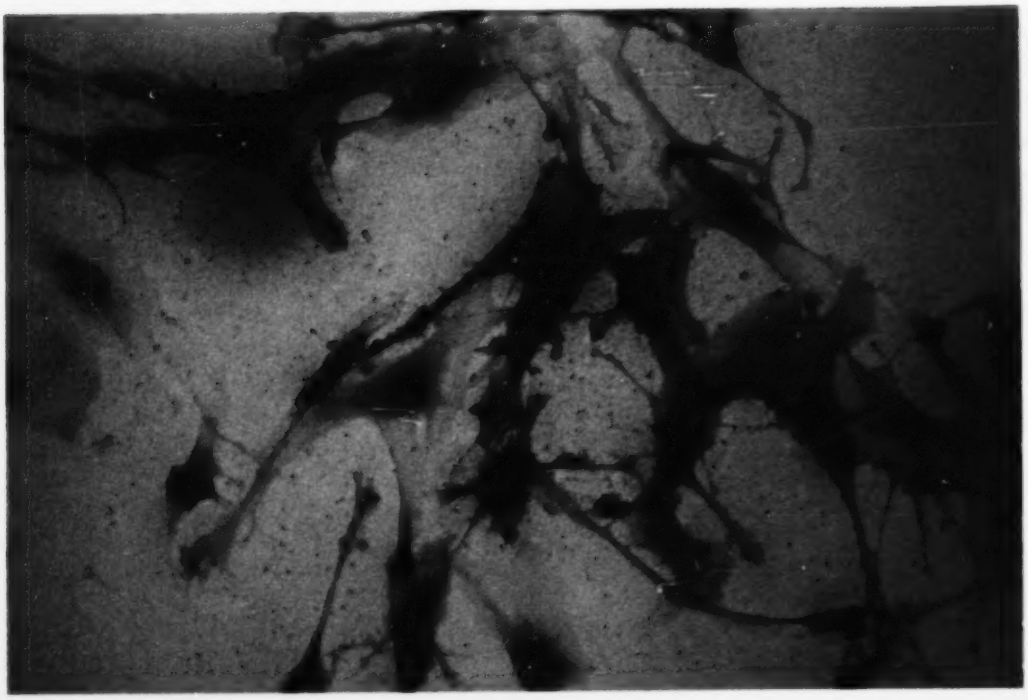


Fig.12. Transduced E-76 cells *in vitro* (X-gal stain).

Fig.13. This section shows the effect of a lower multiplicity of infection(MOI) (100pfu's/ml)of the adenovirus on the transduction rate *in vitro*.The transduced glioma cells appear dark blue while the untransduced cells have taken up the nuclear-fast red counter-stain.

Fig.14. This frozen section shows the site of viral injection into an area of tumour.The extent of transduction is limited to the blue area(arrows ⟶).The injection tract is indicated as shown(**inj** →).The transduced area is bounded by both untransduced tumour(arrow ➡) as well as normal cortex(open arrow ⇨). (X-gal with nuclear-fast red counter-stain).

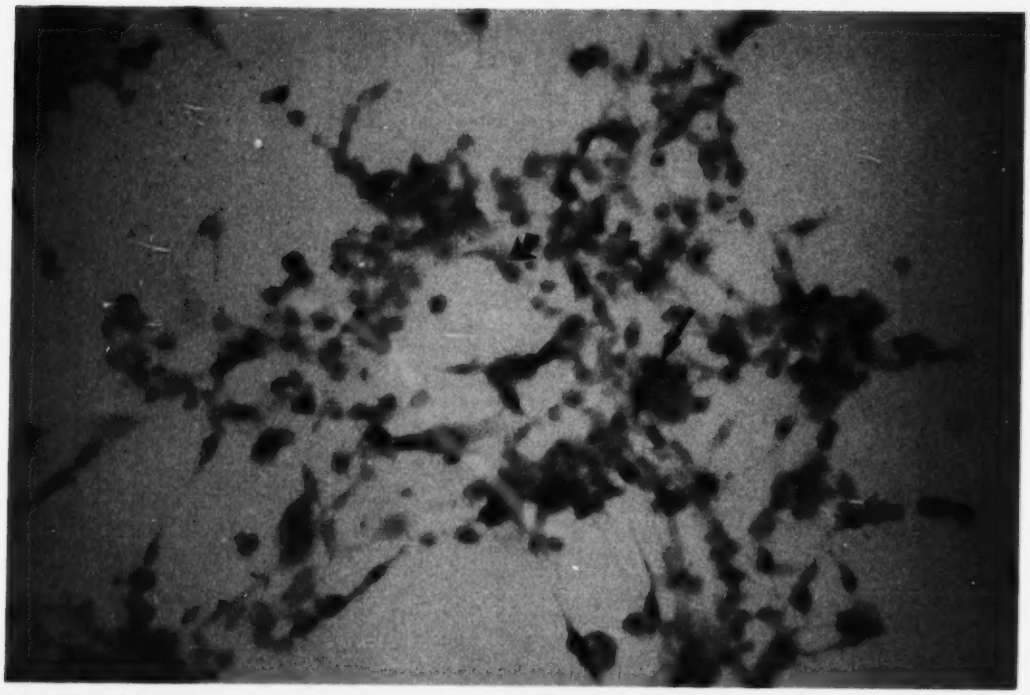


Fig.13. Transduced and non-transduced glioma cells at a lower MOI (X-gal).

Fig.14. Frozen section from F-127 showing injection tract (X-gal).

Fig.15. This frozen section shows the limits of viral transduction which is on average 500-600μM circumferentially around the site of injection.

Fig.16. This frozen section shows two areas of transduced tumour(representing two adjacent sites of viral innoculation)(dark arrows ➡) with an area of untransduced tumour in the center(open arrow ⇨).

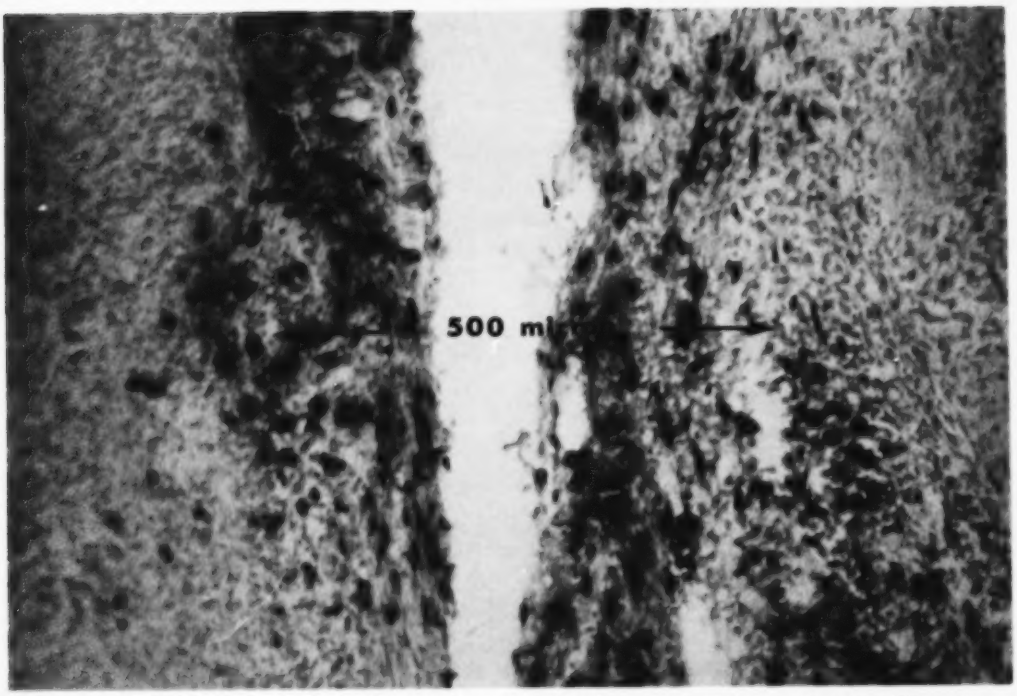


Fig.15. Frozen section from F-127 showing limits of viral transduction(X-gal).

Fig.16. Frozen section showing transduced and non-transduced tumour.

Fig.17. This frozen section shows an area of transduced tumour(small arrows →).

Fig.18. This is a higher power view of Fig.17 showing the transduced blue cells (arrows ⟶).There are untransduced small red cells interspersed with the larger blue transduced nuclei.These may represent pre-pyknotic cells that are resistant to transduction.The overall morphology of these cells is also unlike most glioblastomas in that the cells are more spindle-shaped and tend to be arranged in a fascicular pattern reminiscent of a sarcoma or a mixed gliosarcoma.

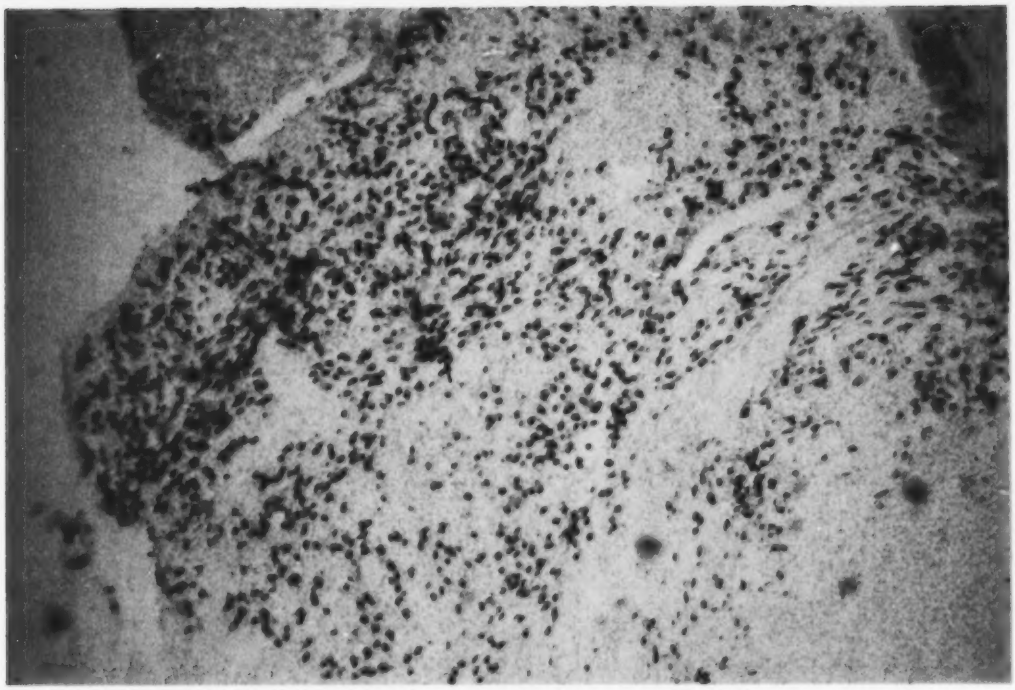


Fig.17. Frozen section of transduced cortical tumour(X-gal).

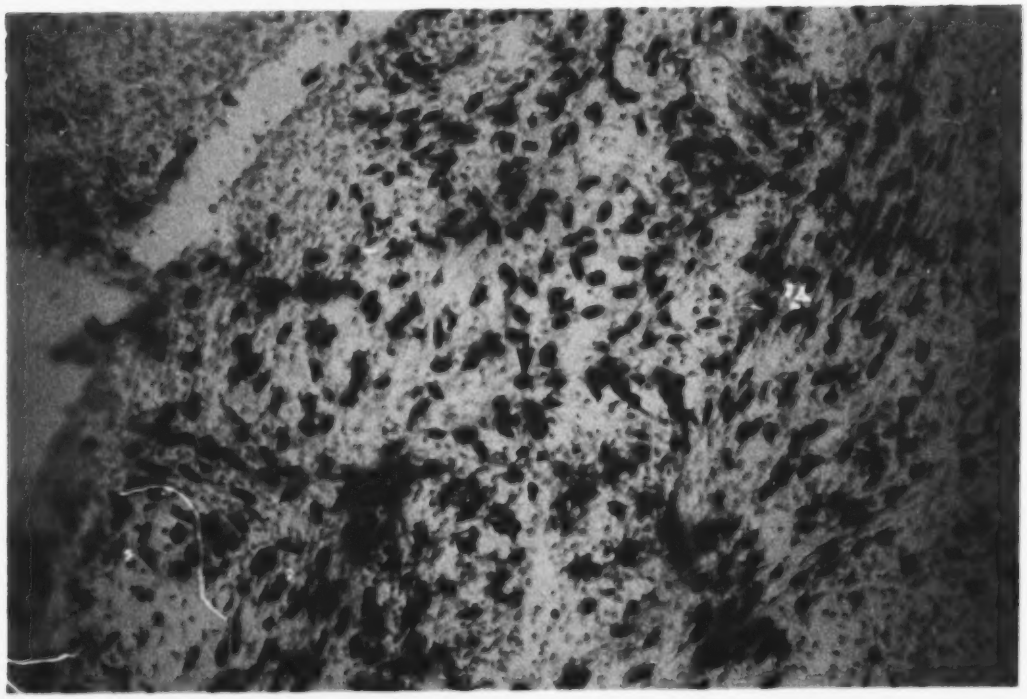


Fig.18. Higher power view of Fig.17.

Fig.19. Frozen section showing an area of surface based tumour which shows excellent transduction with the adenoviral vector(arrows ⟶).

Fig.20. This frozen section is taken from an area of tumour growing along a leptomeningeal surface.Almost all the cells are transduced ,and over a larger area compared to that seen with purely intra-cortical tumours. ➡

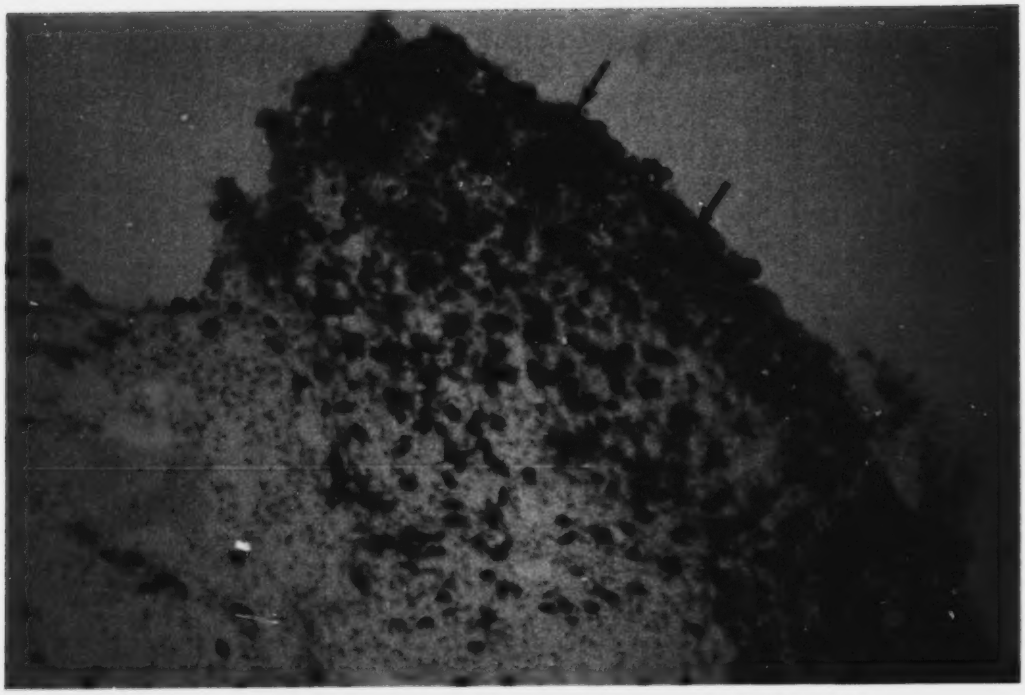


Fig.19. Frozen section showing transduced surface tumour (X-gal).

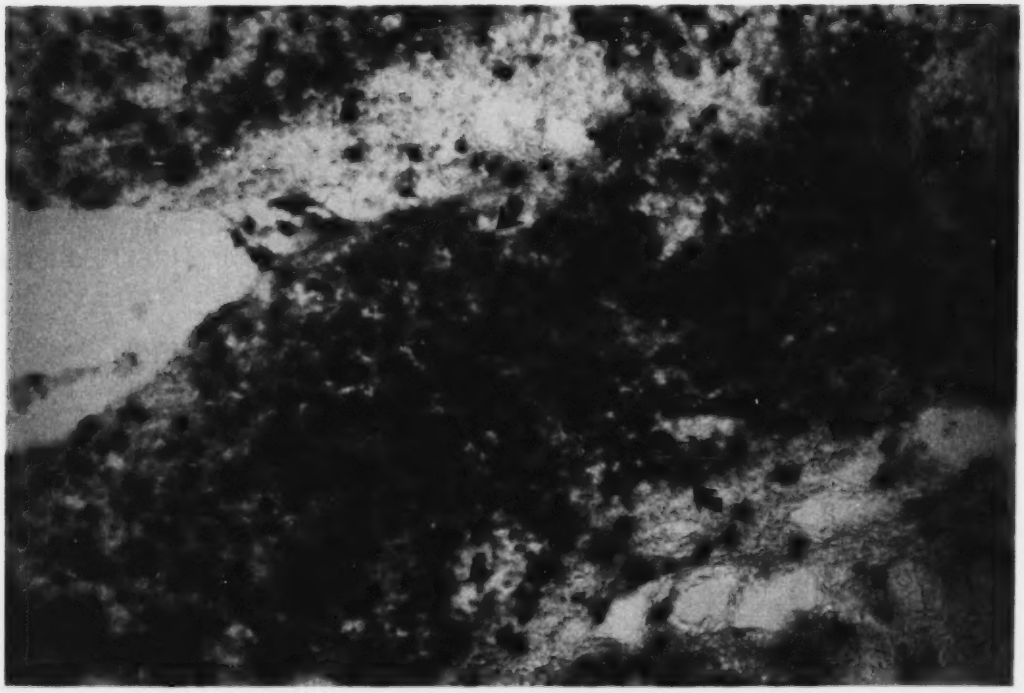


Fig.20. Frozen section of transduced leptomeningeal tumour(X-gal).

Fig.21. Frozen section showing an area of surface tumour with penetration down a sulcus.The X-gal stain shows the adenoviral transduction to be almost wholly confined to the area of tumour(arrow ➡) with sparing of the surrounding normal cortex(open arrows ⇨).

Fig.22. This frozen section represents an area of normal cortex that was injected with the adenoviral vector.The X-gal stain shows that normal neurons and glial cells can be transduced with the virus.

Fig.21. Frozen section showing preferential transduction of tumour .

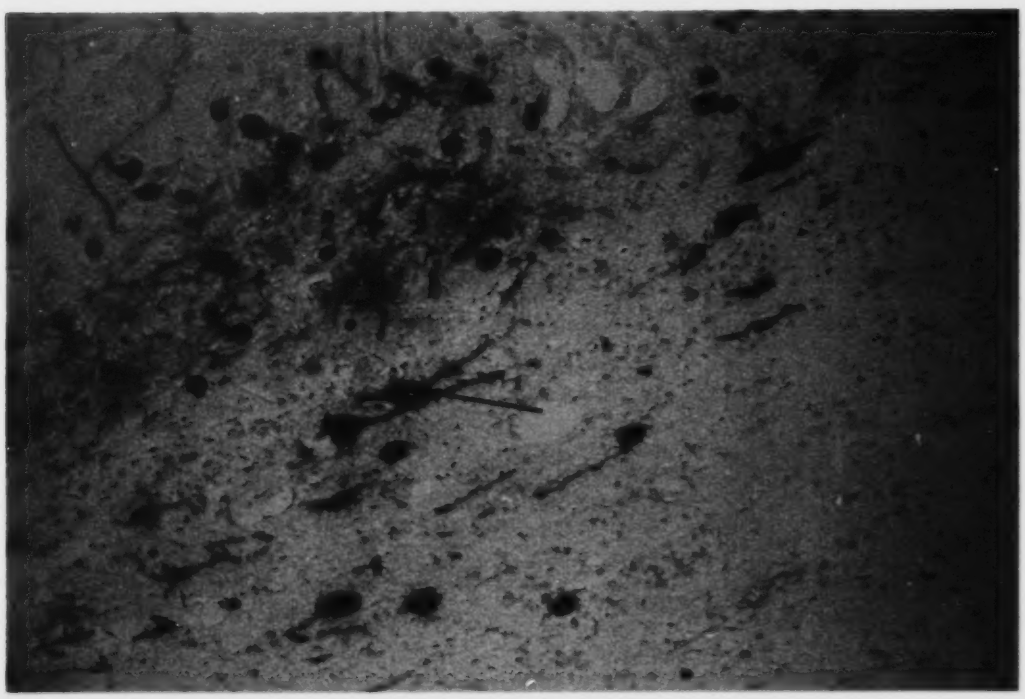


Fig.22. Area of transduced normal cortex following viral innoculation.

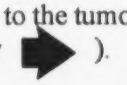
Fig.23. This frozen section highlights the problem with the current method of adenovirus delivery to the tumour cells.Large areas of tumour remain untransduced(arrow ).

Fig.24. This frozen section shows the preferential transduction of some tumour cells with sparing of others within that same area(arrow).This suggests that different tumour cells may show varying degrees of sensitivity to adenoviral transduction.Some of the possible reasons for this selectivity are explored in detail in the accompanying text.

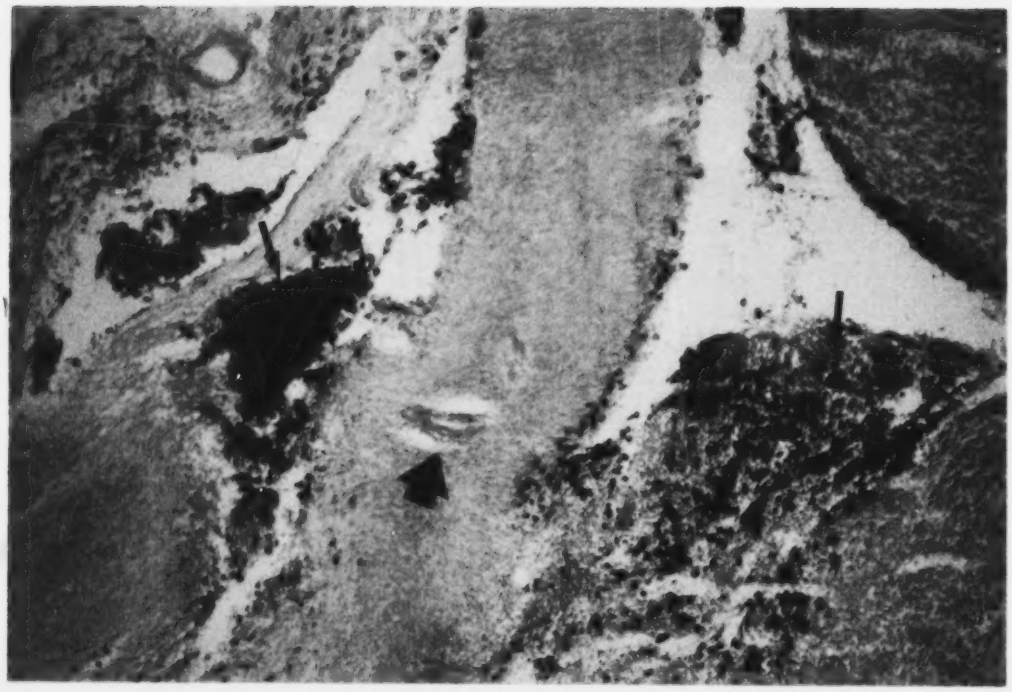


Fig.23. Low power view showing the transduction inefficiency seen with the current mode of viral delivery.

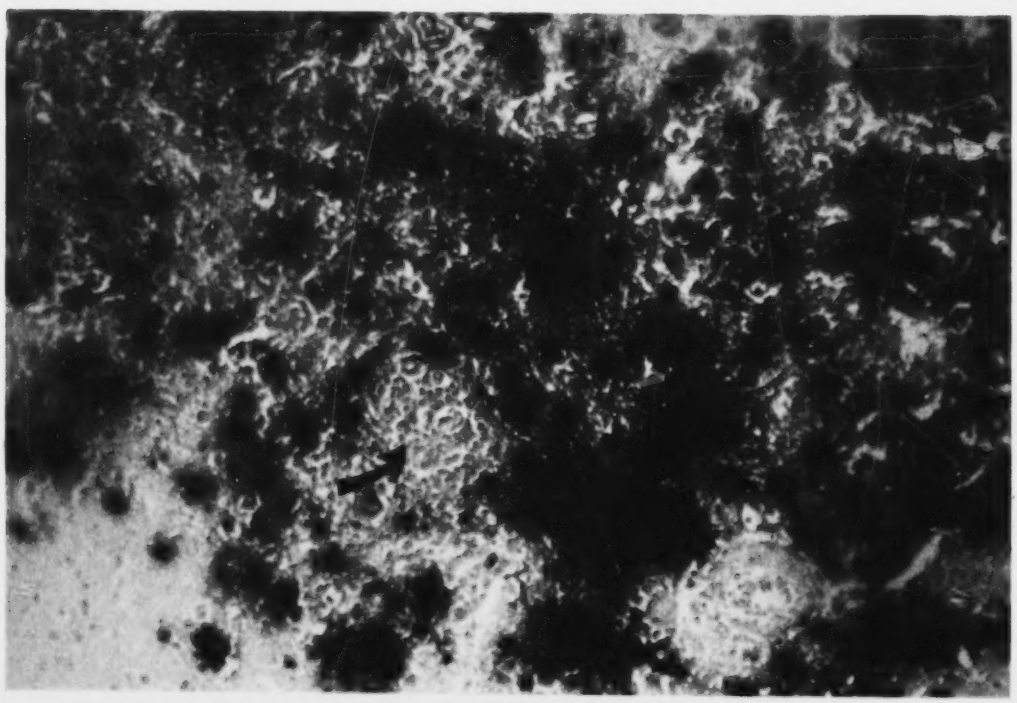


Fig.24. Frozen section showing preferential transduction of some tumour cells.